Volume 110 Number 2

UNIVERSITY OF MICHIGAN
MAY 8 - 1956
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THE BIOLOGICAL BULLETIN

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ERRATUM

AUTHORS' ERROR. Lauffer and Swaby, Vol. 108, pp. 294 and 295.

The dimensions of the lobster hemocyanin molecule, considered as a flattened ellipsoid of revolution hydrated to the extent of .15 ml. water per g. protein, were erroneously reported as 32.2×10^{-7} cm. for the diameter and 7.8×10^{-7} cm. for the thickness. The correct values are 21.4×10^{-7} and 5.2×10^{-7} cm. These are not very close to the electron microscope values. Therefore, the question of the interpretation of the hydrodynamic results is re-opened.

An additional possibility is that the hemocyanin molecules are spheres. If so, the friction ratio of 1.25 must be ascribed to hydration alone. By methods described by Lauffer and Bendet (1954), one can calculate a value of .70 ml. of water per g. of protein from this friction ratio. A sphere composed of protein with partial specific volume of .74, with an anhydrous molecular weight of 825,000 and with this degree of hydration, would have a diameter of 15.6×10^{-7} cm. Such a particle could collapse into a disk upon drying, but its total volume would be less than one-fourth that indicated by the electron micrographs. The new value for hydration is considerably higher than that calculated from the sedimentation vs. density experiments. As was pointed out in a reference cited in the discussion, however, the value calculated from sedimentation vs. density experiments represents only the water in excess of that associated with other constituents in the hypothetical hydrating medium and might underestimate the total.

If one takes the dimensions indicated by the electron micrograph at face value, the particles seen in the micrograph cannot be interpreted as single hemocyanin molecules, for their volumes are much too great. However, if the thickness of the uranium coat is taken into account, the resulting dimensions yield a volume which may not be inconsistent with the hydrodynamic data.

Vol. 110, No. 2 April, 1956

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MOLTING AND SHELL GROWTH IN BALANUS AMPHITRITE NIVEUS[1]

JOHN D. COSTLOW, JR. AND C. G. BOOKHOUT

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The frequency of molting over an extended period of time has not been determined for any barnacle except *Balanus improvisus* (Costlow and Bookhout, 1953). Previously a few estimates had been made on barnacles of unknown age for limited periods only. Darwin (1854) mentioned that Mr. W. Thompson of Belfast maintained 20 specimens of *Balanus balanoides*, for 12 days, presumably in in the laboratory, and on the 12th day all had molted once and one individual twice. Thomas (1944) reported that *Balanus perforatus* molted every fifth day but did not give the age or the length of time these were observed. Costlow and Bookhout (1953) have shown that *B. improvisus* maintained in the laboratory molt every 2–3 days. Several series were followed over various lengths of time, the longest being 120 days during which time the individuals underwent 42 molts. Shell growth was continuous but erratic, and there was no indication that it was related to the molting cycle.

Since *Balanus improvisus* molts more frequently than was suspected by Darwin (1854) and Thomas (1944), and is primarily a winter barnacle at Beaufort, N. C., it may or may not be typical of other species. Therefore, it was deemed advisable to make similar studies on *Balanus amphitrite niveus*, a barnacle which settles during the summer and is most commonly found in the intertidal region at Beaufort. The purpose of this investigation was to determine the molting frequency, period of intermolt, relationship of shell growth to the molting cycle, and the effect of different light conditions.

Methods

One-day old barnacles were collected by suspending plastic squares 6″ × 6″ in the Beaufort Harbor for 24 hours. Small pieces of plastic containing one pinhead barnacle each were cut from the large squares and placed in plastic compartmented boxes. The diet consisted of *Chlamydomonas sp.*, a type of food which supported the best and most consistent growth in *B. improvisus*. All series were maintained in a culture cabinet which was lighted with daylight fluorescent lamps at a tempera-

[1] These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and Duke University, NR 163-194.

TABLE I

Culture conditions for eight series of Balanus amphitrite niveus

Series	Original number	Final number	Days maintained	Light period	Purpose
I	107	18	47	24 hrs. light	molting
II	27	13	47	24 hrs. light	molting and shell growth
III	20	12	18	24 hrs. light	molting and shell growth
IV	35	19	25	24 hrs. darkness	molting
V	32	19	24	24 hrs. darkness	molting and shell growth
VI	40	10	22	12 hrs. light 12 hrs. darkness	molting
VII	20	11	18	12 hrs. light 12 hrs. darkness	molting and shell growth
VIII	56	37	14	natural environment	shell growth

ture of approximately 20° C. The barnacles and surrounding medium were examined daily to determine if a molt were present. The old culture medium was replaced by fresh at this time. Each barnacle studied for growth was measured daily by the use of an ocular micrometer and the areal increment of the basis computed from these measurements for the 24-hour period.

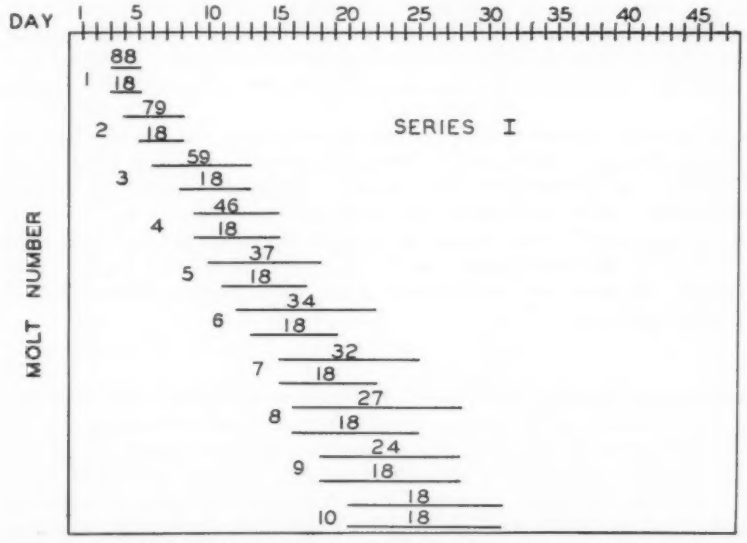

FIGURE 1. Molting periods of *Balanus amphitrite niveus* when reared in continuous light. Series I. The first line indicates the range of time for one molt and the number of molts for entire series. The bottom line indicates the range of time for one molt and number of molts for those individuals which lived throughout the entire period.

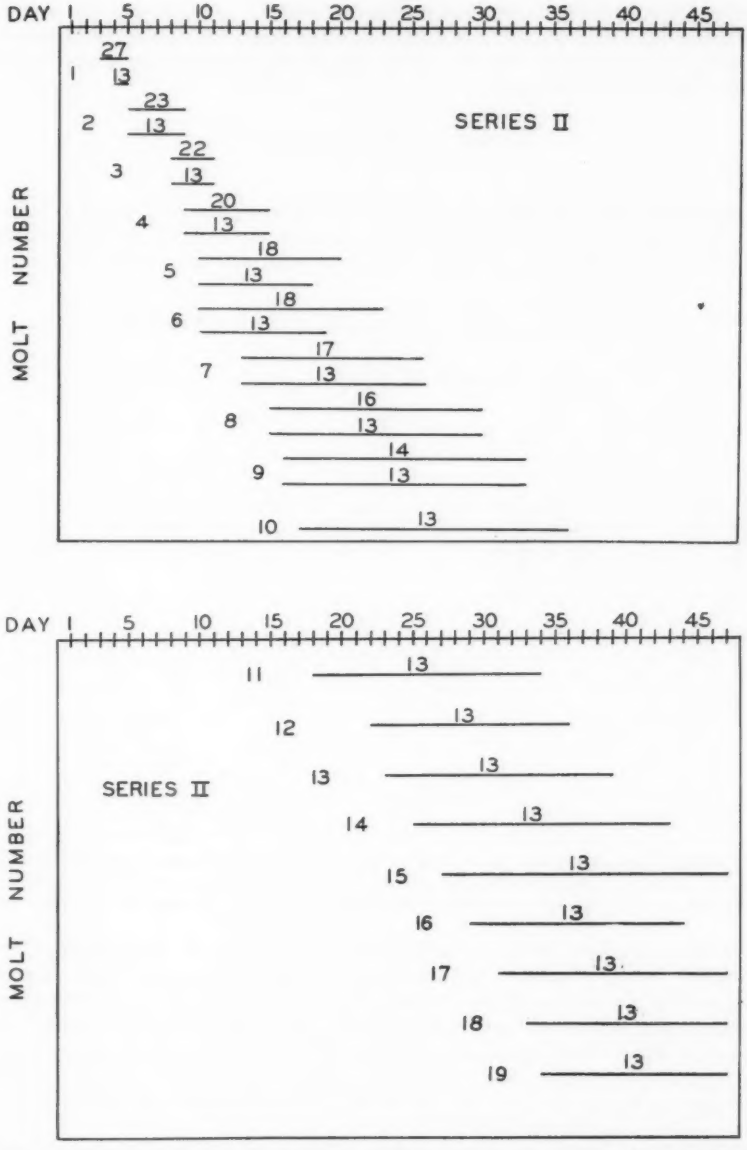

FIGURE 2. Molting periods of *Balanus amphitrite niveus* when reared in continuous light. Series II. The first line indicates the number of molts for entire series. The bottom line indicates the number of molts for those individuals which lived throughout the entire period.

Table I gives pertinent data concerning the number of series and the conditions under which they were raised. In addition to the numbered series, two series were cultured in autoclaved, filtered sea water with no food source, to accurately determine the time of the first molt.

Results and Discussion

Molting

The results of the comparative study of molting are given in Figures 1, 2, and 3. It may be noted that the time of molting for each of the series is relatively uniform for the first five molts, but from the sixth molt through succeeding ones there is

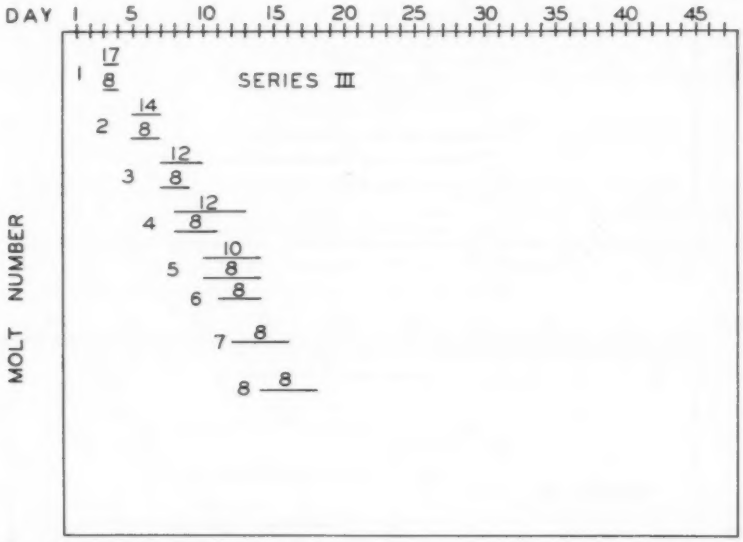

Figure 3. Molting periods of *Balanus amphitrite niveus* when reared in continuous light. Series III. The top line indicates the number of molts for entire series. The bottom line indicates the number of molts for those individuals which lived throughout the entire period.

considerable variation which resembles the condition in *B. improvisus* after the ninth molt. From a study of the individual records of the actual time of molting it is found that the average intermolt period varies from 2–3 days, an interval which agrees with that determined for *B. improvisus*. Extreme variations from this mean were noted within each series, even though they were maintained under identical conditions. For example, some barnacles molted eight times on eight successive days, whereas, within the same series others showed an intermolt period of 5 days over a period of two to three molts.

Figures 4, 5, and 6 show the molting periods for the series reared in total darkness and the series raised in twelve hours of light and twelve hours of darkness. Figure 7 gives a statistical analysis of the molting frequency of barnacles in all

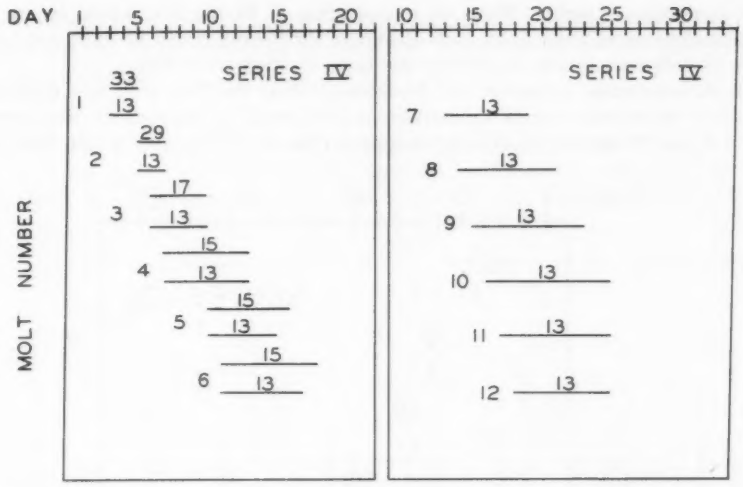

FIGURE 4. Molting periods of *Balanus amphitrite niveus* when reared in continuous darkness. Series IV. The top line indicates the number of molts for entire series. The bottom line indicates the number of molts for those individuals which lived throughout the entire period.

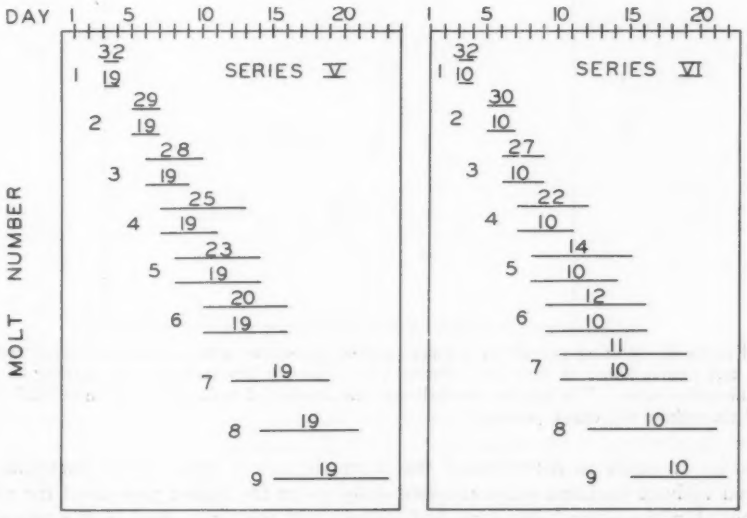

FIGURE 5. Molting periods of barnacles of Series V and VI. Series V was cultured in continuous darkness and Series VI in twelve hours of light and twelve hours of darkness. The top line indicates the number of molts for entire series. The bottom line indicates the number of molts for those individuals which lived throughout the entire period.

three conditions of light. From an examination of Figure 7 it would appear that barnacles grown in total light, total darkness, or twelve hours of light and twelve hours of darkness exhibit essentially the same molting frequency.

In *B. improvisus* (Costlow and Bookhout, 1953) the first molt was overlooked because of the minute size of the exuviae and the masking effect of the food present. The 2–3 day frequency of molting indicated that an earlier molt might have been

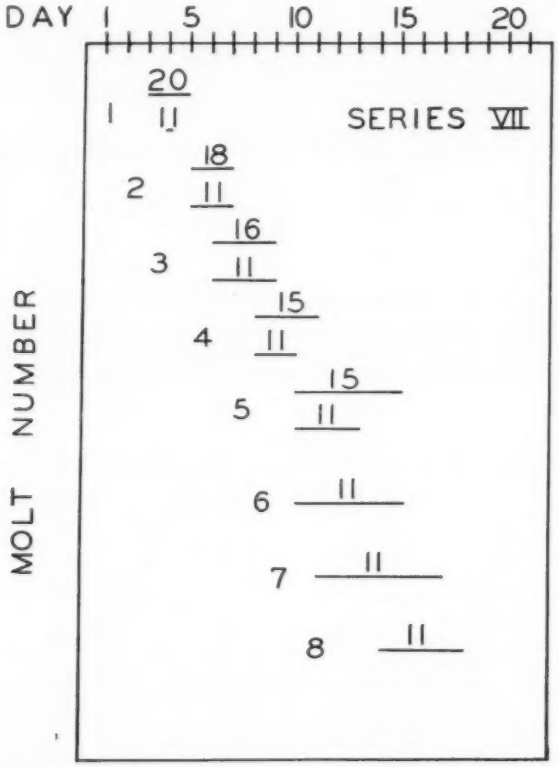

FIGURE 6. Molting periods of *Balanus amphitrite niveus* when reared in twelve hours of light and twelve hours of darkness. Series VII. The top line indicates the number of molts for the entire series. The bottom line indicates the number of molts for those individuals which lived throughout the entire period.

missed. In order to determine if this were the case a series of 10 barnacles was reared without food and was examined daily under the higher powers of the microscope. On the second day nine had molted and the tenth died in the process of molting. This procedure was repeated several times for *B. amphitrite niveus*, but all barnacles died within the first two days without molting. Thus it would appear that *B. amphitrite niveus* will not live nor pass through the first molt without

adequate food. Therefore, it must be concluded from the evidence at hand that the first molt in *B. amphitrite niveus* does not occur until the third–fifth day, the majority on day 4.

Growth

Series II, III, IV, and VII were analyzed for molting frequency and, in addition, the areal increase of each barnacle was computed daily. From the accumulative daily areal increase, as plotted for Series II and IV, it is noted that the

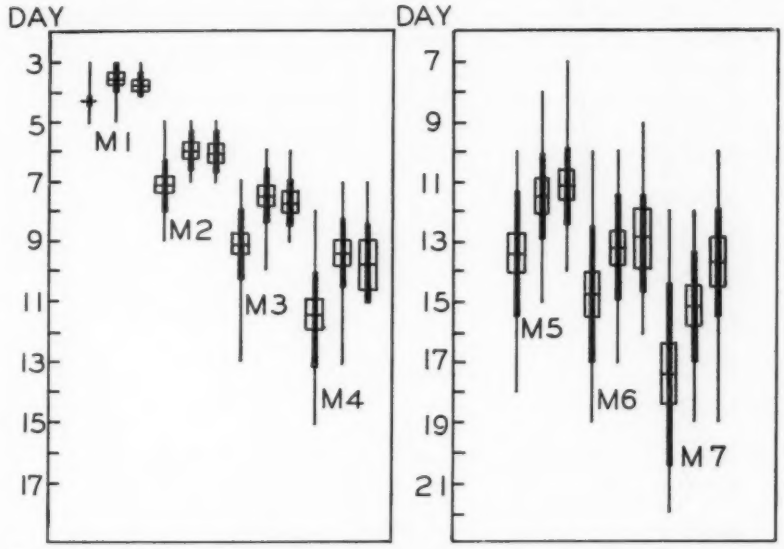

FIGURE 7. Molting frequency of *Balanus amphitrite niveus* under three conditions of light. For every molt a graph is given for each light condition; left line, barnacles exposed to continuous light; middle line, barnacles exposed to continuous darkness; right line, barnacles exposed to twelve hours of light and twelve hours of darkness.

KEY

The vertical line indicates the range.
The solid line indicates one standard deviation on each side of the mean.
The hollow rectangle indicates twice the standard error on each side of the mean.
The cross bar indicates the mean.

growth rate of Series II, grown in 24 hours of light, was less than that for Series IV, grown in continuous darkness (Fig. 8). One might question why shell growth in those cultured in total darkness is greater than in those reared in continuous light. In both conditions CO_2 is given off by the unicellular algae, but in those with continuous light it is utilized in the photosynthetic process. Since this synthesis is curtailed in total darkness the carbon dioxide would accumulate. Thus it would seem that more carbonate would be available to barnacles reared in total darkness and this may conceivably have a direct relationship to shell growth.

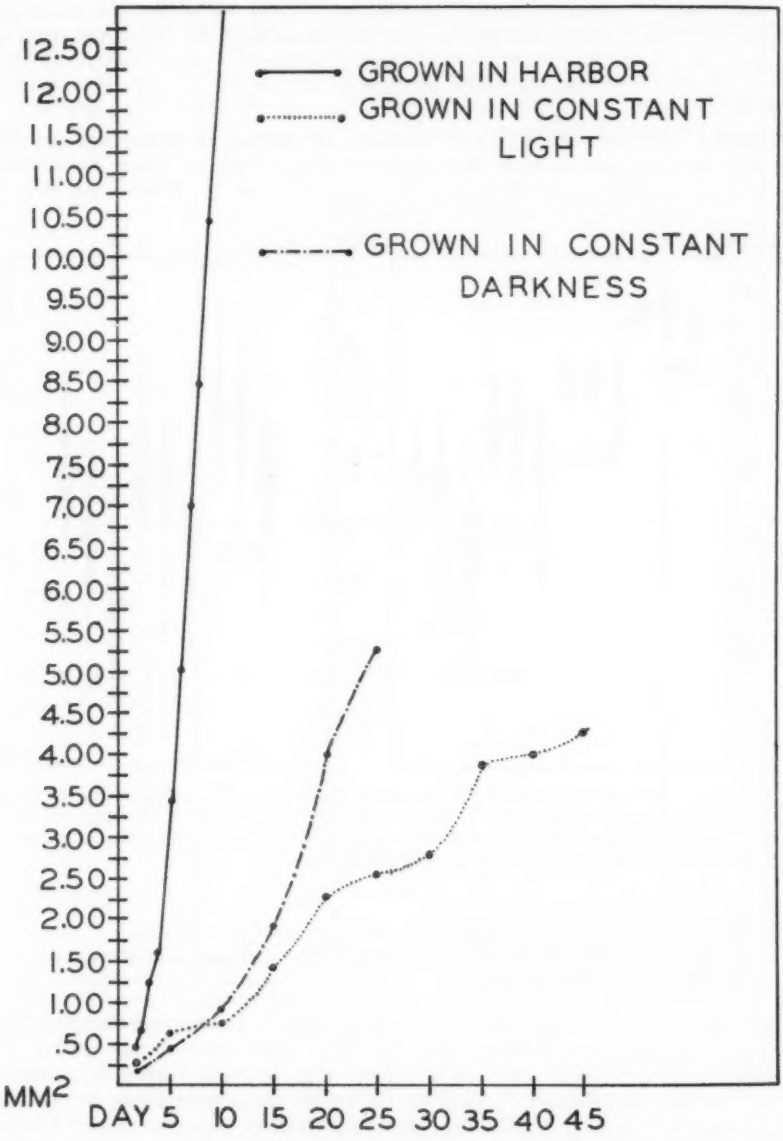

FIGURE 8. Comparison of daily increase in areal diameter in *Balanus amphitrite niveus* reared in continuous light (Series II), continuous darkness (Series IV), and in the natural environment of the harbor (Series VIII).

Periodic fluctuations in shell growth rate were evident in the series grown in continuous light, but not in the series grown in total darkness (Fig. 8), even though they were maintained on the same diet in a constant temperature cabinet. These fluctuations were not evident in *B. improvisus* and the cause in *B. amphitrite niveus* is not known. Unfortunately, Series VII, reared in twelve hours of light and twelve hours of darkness, and the second series grown in continuous light, Series III, could not be maintained beyond 18 days. Thus sufficient data are not available to allow comparisons between these series and the others. Growth in the laboratory, under the conditions described, was never as rapid as that observed for barnacles grown in the harbor.

Mortality

In spite of the uniform conditions provided by a culture cabinet, mortality continued to be very high, even though the barnacles were provided with the same diet which furnished the best growth in *B. improvisus* with practically no mortality

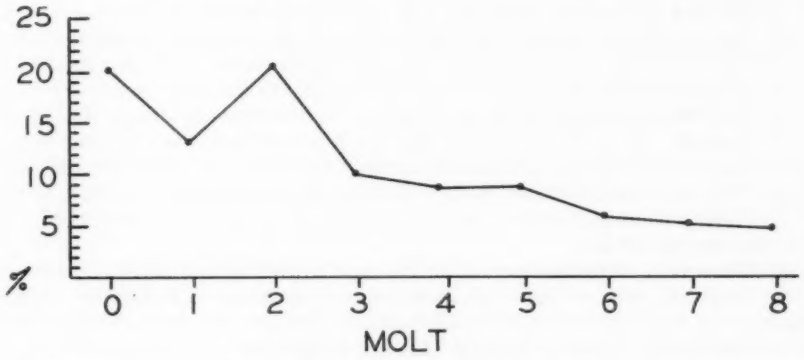

FIGURE 9. Percentage of mortality in relation to molting in *Balanus amphitrite niveus*.

(0.5%). The question arose as to whether death could be due to intrinsic conditions, a particular time in the life cycle, or a specific period within the molting cycle. Examination of Figure 9 indicates that the greater percentage of mortality occurs during the first two molting periods, followed by a steady decrease in succeeding molts. Mortality of *B. amphitrite niveus* in the harbor is also very high, especially during the first week after settling. Records have been kept which show the number of barnacles settled on a 100-cm.^{2} plate over a period of a month. These records include recently settled as well as older individuals for each week of the month. An analysis of plates from June, 1953, indicates that at 5 ft. below mean low water 18 of an original 84 pinheads remained alive after the first week (78.5% mortality). At 6 ft. below mean low water 13 out of 51 survived the first week (74.5% mortality). A portion of this was apparently due to predation since fragments of the whole animal remained attached to the tile after the first week.

In the analysis of molting and growth separate records were made of each individual which lived throughout the entire period. It was believed that these rep-

resented the healthiest barnacles and would more nearly approach those which continue to live in the harbor. An examination of Figures 1–6, giving data for all the barnacles in the individual series as well as those which lived throughout the study, reveals that while the periods for any particular molt are similar for the two groups, the molting periods of the barnacles which continued to live are usually more regular. It is probable that the greater irregularity in the entire series is due to the number of individuals whose metabolism and vigor is reduced and hence, feeding is less than in the more active barnacles. It has been observed that barnacles which molt regularly and live throughout the entire period of study normally cease feeding when disturbed but quickly resume their sweeping activities. Barnacles which show an irregular pattern of molting and shell growth may remain inactive for considerable periods of time after being disturbed.

Conclusions

From a study of 314 *Balanus amphitrite niveus*, reared in the laboratory, the following conclusions may be made:

1. The first molt occurs from day 3 to day 5, the majority on day 4.
2. The second molt occurs from day 5 to day 9, the majority on day 6.
3. The third molt occurs from day 6 to day 13, the majority on day 8.
4. The fourth molt occurs from day 7 to day 15, the majority on day 10.
5. The fifth molt occurs from day 8 to day 18, the majority on day 12.
6. From the 6th to the 19th molt there is little uniformity in the time of any specific molt, resulting in a considerable spread over which one particular molt may occur. This resembles the condition in *Balanus improvisus* after the 9th molt.
7. *Balanus amphitrite niveus* may pass through 19 molts in a period of 47 days after the time of settling.
8. The average intermolt period for *Balanus amphitrite niveus* is 2–3 days.
9. Statistical analysis shows that there is no significant difference in molting frequency in those series reared in 24 hours of darkness, 24 hours of light, or in those reared in 12 hours of light and 12 hours of darkness.
10. Accumulative daily areal increase of basis in a series of barnacles grown in total darkness showed a greater growth rate than the series reared in continuous light.
11. No association between the molting cycle and shell growth, as determined by daily increase in area of basis, was found.
12. *Balanus amphitrite niveus* differs from *Balanus improvisus* in that it will not pass through the first few molts unless adequate food is available.
13. The greatest percentage of mortality in *Balanus amphitrite niveus*, reared in the laboratory, occurs during the first two molting periods and is followed by a steady decrease in mortality through successive molts. In the natural environment of the harbor the highest mortality is found during the first week after settling which corresponds to the condition observed in the laboratory.

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SPAWNING AND EGG PRODUCTION OF OYSTERS AND CLAMS

H. C. DAVIS AND P. E. CHANLEY

U. S. Fish and Wildlife Service, Milford, Conn.

Many aspects of the physiology of reproduction of oysters and clams have been investigated, and most investigators have been impressed by the great number of eggs produced by these animals. Although the number of eggs released, by individual females during a season, is an important factor in determining what constitutes a sufficient breeding stock, this number had never been determined experimentally, and estimates of the number have varied widely.

Brooks (1880) was the first to estimate the number of eggs produced by a female American oyster, *Crassostrea virginica*. His estimate was based on the volume of eggs washed out of ripe females and the calculated volume of a single egg. From the volume calculation Brooks' figures gave 18,750,000 to 125,000,000 eggs, but he believed that as much as 50 per cent should be allowed for foreign matter washed out with the eggs. His estimate, therefore, was nine million eggs per female but states that "an unusually large oyster" gave an estimated 60 million eggs. Churchill (1920) estimated over 16 million eggs and Nelson (1921) estimated 16 to 60 million eggs per female. Galtsoff (1930) observed that individual oysters may release 15 million to 114.8 million eggs at a single spawning and estimated that the maximum number released by a single female during a season may be close to half a billion. Burkenroad (1947), although offering no experimental data, apparently believed Galtsoff's estimate was some ten times too high.

The only known estimate of the number of eggs released by a female clam, *Venus mercenaria*, is that of Belding (1912) who merely states in his summary that the average number of eggs for a 2½-inch quahaug is about two million.

The experiments we are reporting were designed to determine the total number of eggs actually produced by individual oysters and clams under experimental conditions, and to find whether there was any correlation between the size of the individual and the number of eggs produced. We also hoped to find whether this total was affected by the time interval between spawnings, or by the number of times the female spawned.

For our first experiment, 75 oysters were brought into the laboratory on December 30, 1954, and placed in conditioning trays of running sea water at 10.0°–13.0° C. The temperature of the sea water in the trays was raised to 20.0°–21.0° C. on the 31st of December. The clams were obtained on January 5, 1955 and 75 individuals were placed in running sea water at 18.0°–19.0° C. (Loosanoff and Davis, 1950).

In mid-January, after approximately two weeks at conditioning temperature, we started spawning 25 of the clams and 25 of the oysters at three-day intervals. A second group of 25 oysters was spawned at five-day intervals and a third group of 25, at intervals of seven days. The second group of 25 clams was spawned at in-

tervals of seven days and the third group of 25 clams, at intervals of 14 days. To determine the number of eggs produced by each female, each individual was placed in a separate spawning dish, and the shells of the females were marked with a sex symbol and an individual number. The males were discarded as soon as they were identified. The number of eggs released by each female was determined and recorded each time she spawned (Tables I through VI).

The spawning records of the oysters subjected to spawning stimuli every three days are shown in Table I. Within this group the total number of eggs per female ranged from 7.8 million to 59.9 million, while the number released at a single spawning ranged up to 28.3 million. The number of spawnings per female ranged from two to 16. Only number 14 failed to spawn on two or more consecutive three-day trials, and number 10 spawned repeatedly at three-day intervals. The highest number of eggs (59.9 million) was produced by a female that spawned nine times. The female that spawned 16 times ranked second, and a female that spawned only five times ranked third. The lowest total number of eggs was released by a female that spawned seven times. Tables II and III show similar data for the groups spawned at five- and seven-day intervals.

TABLE I

Number of eggs (in millions) released by individual female oysters induced to spawn at 3-day intervals

Days after initial spawning	Female number													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
0	9.0	2.0	6.8	7.9	6.3	0.4	<0.1	—	—	—	—	—	—	—
3	1.0	0.8	—	3.8	8.7	0.5	2.0	0.9	—	—	—	—	—	—
6	3.1	8.4	—	—	—	—	6.8	2.9	<0.1	<0.1	—	—	—	—
9	—	—	0.4	6.9	16.3	2.5	3.3	1.3	—	0.1	1.8	3.3	—	—
12	2.8	18.7	—	1.6	—	1.1	7.7	2.9	—	4.0	2.7	2.3	—	—
15	—	—	—	—	—	—	—	—	—	9.2	—	—	—	—
18	3.9	5.8	—	4.7	—	1.5	—	2.0	7.1	6.5	—	—	5.6	—
21	—	—	—	—	—	—	9.7	—	—	5.6	—	—	1.8	—
24	3.8	—	—	3.5	—	1.8	—	3.6	—	4.1	28.3	16.5	3.6	—
27	0.9	9.6	—	—	10.1	0.02	7.0	2.3	—	4.0	—	0.1	3.4	—
30	—	—	—	—	—	—	—	—	—	2.8	—	—	—	—
33	4.0	—	—	—	—	—	—	6.6	—	—	—	—	6.5	—
36	—	—	—	—	—	—	—	—	—	5.9	—	—	—	—
39	1.0	—	1.4	—	—	—	3.5	—	—	3.6	—	—	3.5	—
42	—	9.4	0.5	3.7	—	—	—	—	—	2.0	1.2	—	1.7	8.4
45	0.3	—	—	—	—	—	—	—	27.3	2.4	—	—	1.4	—
48	—	—	—	—	—	—	—	—	—	—	—	—	—	—
51	—	5.2	—	—	—	—	—	3.2	—	2.7	9.1	—	1.5	—
54	—	—	—	—	—	—	—	—	—	0.6	—	—	—	—
57	—	—	—	—	—	—	—	—	—	0.2	—	—	—	—
60	—	—	—	—	—	—	—	—	—	—	—	—	—	—
63	—	0.03	1.9	—	—	—	—	—	—	—	—	—	—	2.1
66	—	—	<0.1	—	—	—	—	—	—	—	—	—	—	—
Total eggs	29.8	59.9	11.0	32.1	41.4	7.8	40.0	25.7	34.4	53.7	43.1	22.2	29.0	10.5

The record for clams spawned every three days (Table IV) shows a range from 17.1 to 37.4 million in the total number of eggs released. The highest number released by any female in this group at a single spawning was 17.7 million. The num-

TABLE II

Number of eggs (in millions) released by individual oysters induced to spawn at 5-day intervals

Days after initial spawning	Female number													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
0	0.7	0.7	—	—	—	—	—	—	—	—	—	—	—	—
5	—	5.2	0.2	—	—	—	—	—	—	—	—	—	—	—
10	3.4	—	1.2	1.6	1.0	6.8	7.0	3.4	0.1	—	—	—	—	—
15	—	—	5.9	1.1	2.9	—	—	5.5	9.5	—	—	—	—	—
20	—	—	—	3.1	—	—	—	8.4	—	—	—	—	—	—
25	10.0	—	11.4	—	5.4	—	—	—	10.4	10.1	7.0	—	—	—
30	11.8	32.8	10.6	9.1	1.9	15.5	12.6	8.4	6.6	—	—	2.6	35.6	9.3
35	—	—	—	3.0	0.6	—	—	1.7	—	9.2	—	—	—	—
40	7.4	—	16.1	1.8	0.1	—	—	—	—	—	—	—	—	—
45	1.5	8.9	—	—	—	—	0.7	—	1.2	—	—	—	11.0	—
50	—	—	1.2	—	—	—	—	0.3	—	4.3	—	—	—	—
55	6.0	4.2	—	—	—	10.7	—	—	0.3	—	—	9.1	—	10.8
60	—	—	—	—	—	2.3	—	—	0.2	—	—	0.8	—	—
65	0.9	0.4	<0.1	—	—	—	—	—	—	0.2	—	<0.1	—	—
70	0.2	<0.1	—	—	—	—	—	—	—	—	—	—	—	—
75	0.1	—	—	—	—	—	—	—	—	—	—	—	—	—
Total eggs	42.0	52.2	46.6	19.7	11.9	35.3	20.3	27.7	28.3	23.8	7.0	12.5	46.6	20.1

ber of spawnings per female ranged from three to 10, and the female producing the highest total number of eggs spawned seven times. Second highest total was by a female spawning nine times, and the third ranking female spawned only three times. The lowest total was by a female that spawned five times. Tables V and VI give similar data for the clams spawned at seven-day and at 14-day intervals.

The three groups of oysters, spawned at different intervals, were compared after arranging the females according to their rank in egg production (Table VII). The range in total number of eggs per female was from 10 thousand to 66.4 million.

TABLE III

Number of eggs (in millions) released by individual oysters induced to spawn at 7-day intervals

Days after initial spawning	Female number														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
0	0.9	0.8	0.2	—	—	—	—	—	—	—	—	—	—	—	—
7	0.8	0.4	—	12.6	—	—	—	—	—	—	—	—	—	—	—
14	21.0	22.2	5.4	10.1	22.0	0.5	0.3	0.2	0.7	0.4	—	—	—	—	—
21	—	—	—	—	—	—	3.1	—	—	1.0	1.4	7.7	0.4	—	—
28	19.4	39.0	—	—	5.7	—	—	23.4	8.6	—	14.2	—	—	—	—
35	7.7	1.6	10.3	—	—	—	17.0	—	2.5	8.4	25.2	—	9.0	0.5	—
42	1.8	1.9	0.9	4.0	—	48.8	—	—	—	0.7	6.2	12.2	1.4	—	0.01
49	0.6	—	—	<0.1	—	—	—	13.8	—	—	—	—	—	—	—
56	—	0.5	—	—	0.6	3.3	—	—	—	—	3.2	—	—	—	—
63	<0.1	—	—	—	<0.1	—	—	—	—	—	0.1	—	—	—	—
70	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Total eggs	52.2	66.4	16.8	26.7	28.3	52.6	20.4	37.4	11.8	10.5	50.3	19.9	10.8	0.5	0.01

TABLE IV

Number of eggs (in millions) released by individual clams induced to spawn at 3-day intervals

Days after initial spawning	Female number										
	1	2	3	4	5	6	7	8	9	10	11
0	0.3	7.7	1.1	9.3	4.9	8.5	13.6	5.7	4.7	—	—
3	—	—	—	—	—	—	—	—	—	—	—
6	—	2.1	—	—	—	—	6.4	—	—	13.5	—
9	6.4	—	—	3.5	5.9	3.9	—	—	5.4	1.3	11.1
12	—	—	—	—	—	—	—	—	—	—	—
15	2.1	4.2	17.7	1.6	2.5	2.6	4.0	6.3	1.6	2.3	2.5
18	1.4	—	—	—	—	—	—	—	1.0	—	—
21	0.7	3.5	—	—	4.0	0.1	—	1.9	—	3.1	—
24	1.7	—	10.0	2.4	—	3.4	—	0.7	—	1.1	3.7
27	1.1	—	—	1.0	2.2	1.8	6.3	—	—	—	1.8
30	1.4	—	—	—	—	—	—	—	—	—	—
33	—	—	—	—	1.8	—	—	—	—	1.4	—
36	—	4.2	—	—	—	5.0	—	—	5.0	0.8	1.0
39	—	—	—	—	—	—	—	2.5	—	—	—
42	—	—	—	3.2	3.7	2.2	0.3	—	—	—	—
45	—	—	—	—	0.5	—	—	—	—	—	—
48	—	—	—	—	—	—	—	—	—	—	—
51	3.4	—	—	—	—	—	—	—	—	—	—
54	—	—	—	—	2.4	—	6.6	—	—	—	—
57	—	—	—	—	—	—	—	—	—	—	—
60	—	—	—	—	0.2	1.9	—	—	—	—	—
63	—	—	—	—	—	—	—	—	—	—	—
66	—	—	—	—	—	—	—	—	0.2	—	—
69	—	—	—	—	—	—	0.2	—	—	1.5	—
Total eggs	18.5	21.7	28.8	21.0	28.1	29.4	37.4	17.1	17.9	25.0	20.1

TABLE V

Number of eggs (in millions) released by individual clams induced to spawn at 7-day intervals

Days after initial spawning	Female number												
	1	2	3	4	5	6	7	8	9	10	11	12	13
0	4.1	2.0	3.5	2.8	0.1	—	—	—	—	—	—	—	—
7	—	—	5.2	—	—	10.2	—	—	—	—	—	—	—
14	3.4	17.8	2.7	4.2	—	2.4	—	—	—	—	—	—	—
21	3.2	0.5	0.2	4.4	19.3	3.4	11.4	13.1	14.2	2.4	5.5	—	—
28	—	5.7	7.2	1.5	—	—	—	2.4	—	—	3.7	12.5	—
35	3.1	0.7	0.7	2.7	10.4	0.4	5.6	1.7	—	2.1	—	—	14.7
42	2.0	4.4	1.6	3.0	—	—	0.2	—	—	—	—	—	—
49	2.1	1.7	1.9	1.6	—	5.2	3.6	3.2	18.8	—	11.1	7.5	4.1
56	1.4	1.5	—	1.4	—	0.3	0.7	0.8	—	3.3	1.6	—	—
63	0.8	0.9	—	0.7	—	—	0.6	0.3	—	—	—	—	1.9
70	0.6	0.4	1.0	—	4.8	—	0.1	0.2	—	0.2	1.5	—	0.2
77	—	—	—	—	2.3	—	<0.1	—	—	<0.1	—	—	—
84	0.8	0.1	0.2	0.6	2.3	—	—	—	—	—	—	—	—
91	—	—	—	—	0.3	—	—	—	—	—	—	—	—
98	0.3	—	—	—	—	—	—	—	—	—	—	—	—
Total eggs	21.8	35.7	24.2	22.9	39.5	21.9	22.2	21.7	33.0	8.0	23.4	20.0	20.9

TABLE VI

Number of eggs (in millions) released by individual clams induced to spawn at 14-day intervals

Days after initial spawning	Female number													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
0	7.0	—	—	—	—	—	—	—	—	—	—	—	—	—
14	2.0	2.8	21.3	10.0	3.3	5.5	20.4	10.5	6.4	2.7	—	—	—	—
28	7.7	7.3	3.3	3.5	22.4	8.0	8.8	8.8	13.3	18.5	19.8	24.3	10.5	0.7
42	3.2	2.7	3.3	5.4	4.2	4.0	4.3	3.2	4.3	1.1	—	2.7	—	8.7
56	1.4	0.5	2.6	1.6	2.2	1.6	1.3	2.0	1.5	8.0	9.3	9.1	3.4	1.5
70	0.2	—	0.4	0.3	1.1	—	1.6	1.2	0.4	2.9	0.7	—	—	0.1
84	—	—	0.3	<0.1	0.3	—	—	0.6	0.3	1.6	—	—	—	0.2
98	0.1	—	—	—	—	0.2	0.1	0.2	—	—	—	<0.1	1.0	—
112	—	—	—	—	—	—	—	—	—	0.5	—	—	—	—
Total eggs	21.6	13.3	31.2	20.8	33.5	19.3	36.5	26.5	26.2	35.3	29.8	36.1	14.9	11.2

Despite the relatively great differences between females, however, the range in all three groups is essentially similar, and the mean number of eggs per female in all three groups is approximately the same. The 14th and 15th ranking females, spawned at seven-day intervals, appear to have abnormally low totals. Except that

TABLE VII

Total number of eggs (in millions) discharged by individual oysters spawned at 3-, 5-, or 7-day intervals, and by individual clams spawned at 3-, 7-, or 14-day intervals. In each group the females are ranked according to egg production

Oysters				Clams			
Rank	3-day spawning interval	5-day spawning interval	7-day spawning interval	Rank	3-day spawning interval	7-day spawning interval	14-day spawning interval
1	59.9	52.2	66.4	1	37.3	39.5	36.5
2	53.6	46.6	52.6	2	29.5	35.7	36.1
3	43.1	46.6	52.2	3	28.8	33.0	35.3
4	41.3	42.0	50.3	4	28.2	24.2	33.5
5	40.0	35.3	37.4	5	25.1	23.4	31.2
6	34.4	28.3	28.3	6	21.7	22.9	29.8
7	32.2	27.7	26.7	7	21.0	22.2	26.5
8	29.8	23.8	20.4	8	20.2	21.9	26.2
9	29.0	20.3	19.9	9	18.7	21.8	21.6
10	25.6	20.1	16.8	10	18.0	21.7	20.8
11	22.2	19.7	11.8	11	17.0	20.9	19.3
12	11.0	12.5	10.8	12	—	20.0	14.9
13	10.4	11.9	10.5	13	—	8.0	13.3
14	7.8	7.0	.5	14	—	—	11.2
15	—	—	.01	15	—	—	—
Mean	31.4	28.1	27.0	Mean	24.1	24.2	25.4
Average per spawning	4.4	5.5	7.2	Average per spawning	3.6	3.6	4.7
Maximum number of eggs from one female at a single spawning 48,780,000				Maximum number of eggs from one female at a single spawning 24,300,000			

they were the last to spawn, spawned only once, and released exceptionally few eggs, there seems to be no reason to exclude them.

A similar comparison of the three groups of clams spawned at different intervals (Table VII) shows that they have even more closely comparable ranges, in the total number of eggs per female, than do oysters. The mean numbers of eggs per individual in the three groups of clams are also in closer agreement.

An analysis of variance test confirms the conclusion that varying the spawning interval from three to seven days for oysters and from three to 14 days for clams did not affect the total egg production. We believe that the similarity of variances and the agreement of means further indicate that each group represented an adequate sample of the populations used.

From Table VII we see that the maximum number of eggs released by one female at a single spawning, in either clams or oysters, may be greater than the seasonal total of many other females in the same population. We also note that the average number of eggs per spawning increases progressively, in oysters, as the interval between spawnings is increased. In clams there is no difference between the groups spawned at three- and seven-day intervals, but the average number of eggs per spawning increases slightly as the spawning interval is increased to 14 days.

If we compare clams with oysters, we find that clams are more uniform than oysters in egg production. The average number of eggs per female varied less from group to group (Table VII), and the range in total number of eggs released by different female clams was not so great. Moreover, as shown by the frequency distribution, the number of female clams producing different total numbers of eggs had a fairly normal distribution with a well-defined modal class at 20 to 24.9 million eggs per female, and the arithmetic mean number of eggs per female (24.6) falls within this class. In oysters, by contrast, the distribution was not obviously a normal one, and there is no well-defined modal class near the arithmetic mean (28.8 million). A comparison of the frequencies of individual spawnings in which different numbers of eggs were released also illustrates the greater variability of oysters since, again, the clams have a lesser range and a somewhat better-defined modal class.

The average number of spawnings per oyster decreases progressively as the interval between spawnings increases, while with clams there is little difference among the three groups. An analysis of variance confirms the conclusion that varying the spawning interval for oysters significantly affects the number of times a female oyster will spawn, with those subjected to spawning stimuli at shorter intervals spawning more frequently. The analysis also shows that varying the spawning interval from three to 14 days had no significant effect on the average number of spawnings per individual clam.

To find whether any correlation existed between the size of the female oyster and the number of eggs produced, we chose the volume of the shell cavity as our best criterion of size. The total number of eggs produced, expressed in millions, was then plotted against the volume of the shell cavity in ml. for each female (Fig. 1). In plotting, different symbols were used to differentiate the groups spawned at different intervals. The plot shows that, while the three separate correlations would differ, there is no striking reversal of trend. The data were therefore combined, and one over-all correlation was computed for all oysters in the experiment

considered as a single group. The resulting correlation was reasonably good: r was .54 (significant at the .01 level for 41 degrees of freedom), which means that about 30 per cent of the variation in total egg production for oysters could be attributed to the differences in size as denoted by cavity volume.

For clams a similar plot of the number of eggs in millions was made against the shell cavity volume in ml. (Fig. 2). Again, we used different symbols for the groups spawned at different intervals, but the data were combined for computing an over-all correlation. For clams, r was .38 (significant at the .05 level for 36 degrees of freedom), which means that about 15 per cent of the variation in total

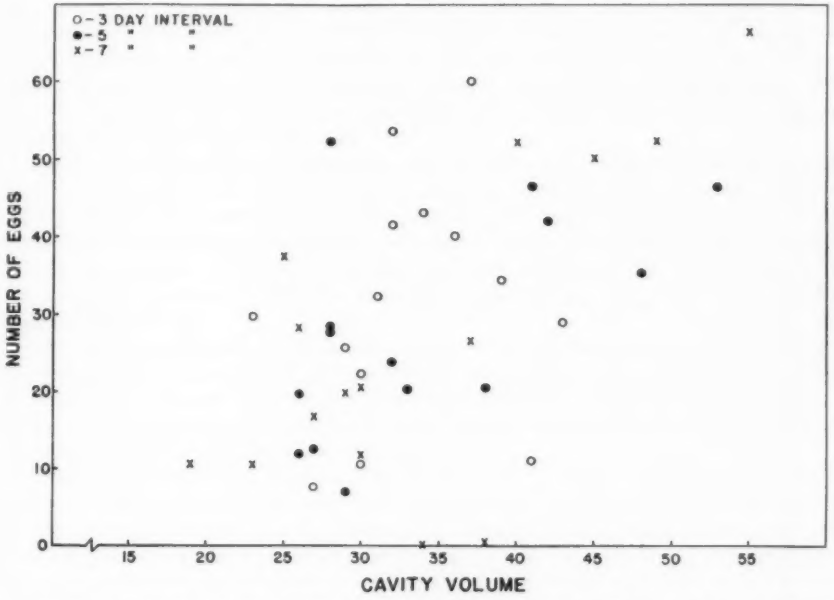

FIGURE 1. Scatter diagram showing the total number (in millions) of eggs produced, plotted against the volume (in milliliters) of the shell cavity, for each of 43 oysters. Different symbols are used to differentiate between females spawned at 3-, 5-, and 7-day intervals.

egg production could be attributed to the differences in size of the female clams used in the experiment.

A similar test was made for a correlation between the number of times a female spawned and the total number of eggs produced. The correlation for oysters was .51 (significant at the .05 level). Thus, in general, females that have a large number of eggs to release will spawn at more frequent intervals than females having a lesser total number of eggs. For clams this correlation was only .17, or was not significantly different from zero.

It should be remembered that the oysters and clams used in this experiment were brought into the laboratory in mid-winter and that conditioning, while in

trays of running sea water, does not provide optimum feeding conditions. Field observations in Long Island Sound have shown that, in some seasons, oysters that go through the winter with relatively little glycogen may increase this reserve by as much as two or three times in the spring before gonad development begins. Moreover, Loosanoff and Nomejko (1951) showed that the average gonad thickness of oysters in Long Island Sound, at the beginning of some spawning seasons, may be almost double that recorded at corresponding periods of other years. Although no measurements of gonad thickness or glycogen content were taken at the

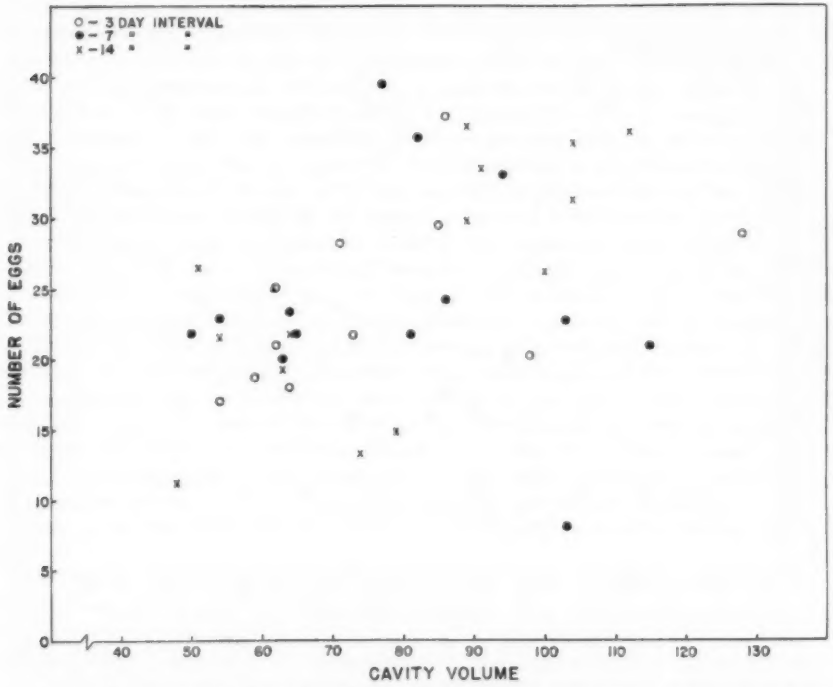

FIGURE 2. Scatter diagram showing the total number (in millions) of eggs produced, plotted against the volume (in milliliters) of the shell cavity, for each of 38 clams. Different symbols are used to differentiate between females spawned at 3-, 7-, and 14-day intervals.

beginning of the experiment, from inspection we believe that the condition of the oysters used in these experiments was below average for oysters of Long Island Sound.

A similar experiment, involving only nine females, was started on June 27, 1955 with oysters that had developed gonads under normal conditions in Long Island Sound. The total number of eggs per female ranged from 23.2 million to 85.8 million, and averaged 54.1 million eggs per female. Thus, both the average number of eggs and the maximum number per female were about 20 million higher than

in the winter experiment. This was, we believe, at least in part, the result of these oysters having built up additional reserves of glycogen during the spring prior to the initiation of gonad development. Even these oysters, however, gave far fewer eggs than the ½ billion per female suggested by Galtsoff (1930).

From the present studies, and from another experiment in which 70,000,000 eggs were released by one female at a single spawning, we are in general agreement with Galtsoff (1930) on the maximum number of eggs a female oyster may release at a single spawning. Galtsoff's estimate of ½ billion eggs as about the maximum number a female oyster might release in a single season was based, in part, on the assumption that a female might release approximately 100 million eggs at each of five or six spawnings during a season. Our experiment indicates that this would be unlikely. For example, the female that gave the highest number of eggs at a single spawning, 48.8 million, spawned on only two other occasions during the experiment, and on each of these occasions released less than four million eggs. Of the 43 female oysters in the experiment, only one released in excess of 10 million eggs at as many as three spawnings.

At the conclusion of the experiment, all the females were opened and the gonad condition, presence of shell injuries, and degree of Polydora and sponge infestations were noted for each female. Statistical analysis does not reveal any effect of shell injuries, or of Polydora and sponge infestations, on the number of eggs produced by a female oyster. Histology indicated that only one female oyster still contained a few eggs, but that all the female clams still retained a few apparently mature eggs (Loosanoff, 1937).

We were surprised to find that it required about 2½ months, or approximately the duration of a normal spawning season, to completely spawn out either oysters or clams, even though they were subjected to spawning stimuli every three days. Moreover, there was almost no difference in the time required at the different spawning intervals used in this experiment.

There were marked differences, however, between clams and oysters in their behavior when subjected to chemical and thermal stimulation in the spawning dishes. Usually 80 per cent to 100 per cent of the oysters opened within 15 or 20 minutes after being placed in the spawning dishes, and normally they remained open until after spawning or until they were disturbed. Records available on 183 of the 227 spawnings of female oysters, included in the present experiment, showed how long each female was open before starting to spawn. This period ranged from one minute to 219 minutes, with an average of 34.2 minutes. Approximately 80 per cent of the spawnings occurred 30 minutes or less after the female opened, about 10 per cent of the spawnings occurred within 31 to 60 minutes, and about 10 per cent of the spawnings occurred over an hour after the female had opened and started pumping.

Clams, on the other hand, were much less predictable. A few opened almost immediately after being put in the spawning dishes, some opened only after several hours, and usually there were a few that remained closed throughout an attempted spawning. Even after opening they frequently closed again for variable lengths of time for no apparent reason. The interval between the time the clams first opened and the time they started spawning was recorded for 208 of the 235 spawnings in this experiment. This interval ranged from less than five minutes to 840

minutes, with an average of 137 minutes. Moreover, only 35 per cent of the spawnings occurred within one hour after the clams first opened. Both male and female clams have been observed to spawn for a short time, then, while still remaining open, cease spawning for periods of an hour or more, and finally spawn even more heavily than at first. Also, on several occasions both male and female clams that had been open and pumping vigorously for 30 minutes or more started spawning immediately when placed in dishes of fresh, cooler sea water.

TABLE VIII

Spawning record of male and female oysters subjected to spawning stimuli daily for five consecutive days; x indicates spawning

Females						Males					
Female number	Days					Male number	Days				
	1st	2nd	3rd	4th	5th		1st	2nd	3rd	4th	5th
1	x	—	x	—	—	1	x	x	x	x	x
2	x	—	x	—	—	2	x	x	x	—	x
3	x	x	x	x	—	3	x	x	x	—	x
4	x	—	x	x	x	4	x	x	x	x	—
5	x	x	x	x	x	5	x	x	x	—	x
6	x	—	x	x	x	6	x	x	x	—	—
7	x	—	x	—	x	7	x	x	x	x	x
8	x	x	x	x	x	8	x	x	x	x	x
9	x	—	—	x	—	9	x	x	—	—	x
10	x	—	x	x	—	10	x	x	x	—	x
11	x	x	x	x	x	11	x	x	x	x	x
12	x	x	—	—	x	12	x	x	x	—	x
13	x	x	x	x	—	13	x	x	x	—	x
14	x	—	x	x	—	14	x	x	x	—	x
15	x	x	x	—	—	15	x	x	x	—	x
16	x	x	—	—	—	16	x	x	x	—	x
17	x	x	—	x	—	17	x	x	x	—	x
18	—	x	—	—	x	18	x	x	x	x	x
19	—	x	—	x	—	19	x	x	x	x	x
20	—	—	x	—	x	20	x	x	x	—	x
21	—	—	x	—	—	21	x	x	x	x	x
22	—	—	x	x	—	22	x	x	x	—	x
23	—	—	x	—	—	23	x	x	x	x	x
24	—	—	x	—	—	24	x	x	x	—	x
						25	—	x	x	x	—
						26	—	—	x	—	—

As noted in Table I, only one of the 14 female oysters failed to spawn two or more consecutive times when subjected to spawning stimuli every third day, and female number 10 spawned repeatedly at three-day intervals. This led us to suspect that at least some female oysters might not show the two- to five-day refractory period that Galtsoff found (1938, 1940). An experiment was therefore designed to determine whether some female oysters might spawn at more frequent intervals.

For this experiment, 50 oysters were brought into the laboratory April 4, 1955, and placed in conditioning trays at 20.0°–21.0° C. Twenty-one days later these oysters were placed in individual spawning dishes, and both chemical and thermal stimulation were used to induce spawning. Seventeen females and 24 males re-

sponded on the first day (Table VIII). After spawning, the males and females were washed separately in cold running sea water, and the shell of each was marked with the appropriate sex symbol and an individual number. All males were then returned to one conditioning tray, and all the females to another tray. Those oysters that did not spawn on this first trial were returned to a third tray. Each morning, for five consecutive days, all oysters were subjected to spawning stimuli and a record of the spawning of each individual was kept.

As shown in Table VIII, of the 24 females in the experiment, 14 spawned on two or more consecutive days, eight spawned on three or more consecutive days, five spawned on four or more consecutive days, and three spawned on each of the five days of the experiment. As was to be expected, 25 of the 26 males in the experiment spawned on three or more consecutive days, nine spawned on four or more consecutive days, and eight spawned on each of the five days of the experiment. By the fifth day, however, it was difficult to induce spawning and the spawning of both males and females was light. However, when these oysters were again subjected to spawning stimuli, after remaining undisturbed in the conditioning trays for an additional week, 22 of the 26 males and 18 of the 24 females responded, releasing approximately average amounts of spawn.

From our experiments, we believe that either there is no refractory period for female oysters, such as Galtsoff (1938, 1940) described, or it is less than 24 hours in duration. Our results suggest that both male and female oysters can spawn, upon proper stimulation, any time they have physiologically-ripe sex cells to discharge. Under constant, or closely spaced intervals of stimulation, we believe they may so deplete their supply of physiologically-ripe sex cells that they are unable to release more until additional sex cells have become physiologically mature. Some females, for example, were observed to give fairly typical spawning motions on the fourth and fifth days of the above experiment without releasing any eggs and yet, spawned normally after several additional days of conditioning.

We wish to express our thanks to the Director of Milford Laboratory, Dr. V. L. Loosanoff, who suggested this problem, for his advice throughout the experiments, and to our colleagues, Mrs. Barbara Myers, for the statistical treatment, and Mr. C. A. Nomejko, for the figures.

Summary

1. The total number of eggs released by individual female oysters, *C. virginica*, conditioned in mid-winter, ranged from 10 thousand to 66.4 million, and averaged 28.8 million.

2. Females that developed gonads under natural conditions in early summer released from 23.2 to 85.8 million eggs for an average of 54.1 million eggs per female.

3. In this experiment the highest number of eggs released by a female oyster in a single spawning was 48.8 million, but in a previous experiment one female discharged 70.0 million. Thus, some females release more eggs at a single spawning than other females do in a season.

4. There was no significant difference in the average number of eggs released in a season whether the oysters were spawned at three-, five-, or seven-day intervals.

5. Female oysters that had a large number of eggs to release tended to spawn more frequently than females that had a lesser number.

6. The average number of spawnings per female oyster decreased progressively as the interval between spawnings was increased.

7. The total number of eggs produced showed a correlation of .54 (significant at .01 level) with the size of the female oyster, as indicated by shell cavity volume.

8. No correlation could be found between the number of eggs produced and Polydora or sponge infestation, or shell injury.

9. We find no two- to five-day refractory period during which female oysters cannot be induced to spawn, as reported by earlier investigators.

10. The total number of eggs released by individual female clams, *V. mercenaria*, ranged from eight million to 39.5 million, and averaged 24.6 million per clam.

11. The highest number of eggs released by any female clam at a single spawning was 24.3 million.

12. For clams, the correlation between number of eggs produced and volume of shell cavity was .38 (significant at the .05 level).

13. There was no significant difference in the average number of eggs released in a season whether the clams were spawned at three-, seven-, or 14-day intervals, nor was there any significant difference in the average number of spawnings per female.

14. The correlation between the number of times a female clam spawns and the number of eggs produced was not significantly different from zero.

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THE EFFECT OF TEMPERATURE ON TESTICULAR RECRUDESCENCE IN JUNCOS AT DIFFERENT PHOTOPERIODS[1]

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Some, perhaps many, biologists specializing in field observations are unwilling to accept the concept, developed through three decades of experimental work, that length of the daily periods of light and darkness, and their seasonal changes, play a fundamental role in regulating the annual reproductive cycle in birds. Thus, Blanchard (1941, p. 76) deems it ". . . extremely doubtful whether the abundant means which have been discovered for upsetting the physiological balance of captive birds should be accepted as possessing any bearing whatever on the factors which control the cycle under natural conditions." She believes (p. 74) that temperature is the most important single factor lying at the ultimate source of annual variations of the gonad cycle in white-crowned sparrows. Marshall (1951, p. 245) calls for "far more elegant experimental techniques than those used in the past . . . before it can be unquestionably accepted that light fluctuation causes . . . prenuptial sexual recrudescence." He holds (p. 257) that the annual cycle is kept in step with the sun not by the changing photoperiod but by external factors that permit nest building and the events subsequent to nest building, and he includes "mild weather" among the probably most important of these external factors.

The literature dealing with experimental studies of the effect of temperature and day-length on the gonads of birds has been reviewed by Burger (1949). Two papers published prior to that time are especially pertinent to the present study. Burger (1948) found that when starlings are subjected to relatively constant high temperatures (either 32°–35° C. or 38°–40° C.) testicular recrudescence proceeds at a rate faster than when birds are exposed to fluctuating moderate temperatures (11°–24° C., with a daily range of about 6°), providing that the photoperiod is favorable. On the other hand, Kendeigh (1941) found that exposure to fluctuating outdoor temperatures averaging about 2° C. did *not* limit gonad increase under lengthened photoperiod in the English sparrow. More recently Farner and Mewaldt (1952), in a study on white-crowned sparrows of which we have seen only an abstract, conclude (p. 107) that "if photoperiod and light intensity are sufficient to induce early recrudescence, the rate of recrudescence can be accelerated by increased temperature."

The experiments now to be reported do not have the "elegant" design called for by Marshall, but they are the first to test the effect of moderately high and low temperatures on testicular recrudescence in birds exposed to different photoperiods near the supposedly critical or minimum limit. For an understanding of the relative importance of these two factors, temperature and day-length, in the timing of

[1] This investigation was supported in part by a research grant (E-356) from the National Microbiological Institute of the National Institutes of Health, Public Health Service.

the early phases of seasonal reproduction it seems especially important to study their interaction at levels near those occurring in nature at the time these early gonad changes are taking place. The importance of approaching the subject in this way seems obvious, yet in the much-studied field of bird photoperiodism no such study has been made heretofore. The results, we hope, may promote reconciliation of some opposing views and apparently contradictory statements to be found in the literature.

Materials and Methods

Sixty-six male Juncos and two females were used in the experiments. Presumably of the race *Junco hyemalis hyemalis* (Miller, 1941; pp. 321, 329), all were trapped or netted in the immediate vicinity of Chapel Hill, N. C. The birds of groups G, H and J (Table I) were taken in late November and (mostly) in December, 1954; those of groups A through F were captured in January and February (A, B and C in 1954; D, E and F in 1955). Birds collected at any one time were assigned at random to the several groups by the turning of cards. (Scatter diagrams of the results indicate no correlation with date of capture.) Each group of birds was confined in a cage made of ¼ inch-mesh hardware cloth and measuring 36 in. × 24 in. × 24 in.; they were fed a commercial chick mash (Purina Growena). Until experimental lighting was begun (Table I, "Dates"), the birds were held in an unheated attic near a small, north-facing window, hence on nearly natural day-lengths. For the experiments, the cages were placed each in a light-tight, ventilated compartment measuring approximately 60 in. × 48 in. × 48 in., lighted by two 100-watt white-color fluorescent tubes. The lights were operated by automatic timer switches; they delivered approximately 150 foot-candles of light on the floor of the cage. A Taylor maximum-minimum thermometer was placed beside each cage and the temperatures recorded daily. Groups A through F were held on a 10-hour photoperiod until the middle of April; then, for six weeks they were exposed to the test photoperiod (10, 11 or 12 hours), certain of the groups being transferred for this test period to a cold-room maintained at 4°–8° C. Group C was placed in the cold-room during the dark period only, and removed daily to a relatively warm room for the lighted period. At the end of the test period the birds were sacrificed and the testes fixed *in situ* in Helley's fluid. Subsequently, after one week in 80% alcohol, the testes were measured, under a binocular dissecting microscope, with vernier calipers reading to 0.1 mm. From these measurements volumes were calculated according to the rule for a prolate spheroid. Later one testis of each bird was excised and eventually sectioned for determination of spermatogenic state and measurement of tubule diameter.

We chose photoperiods of 12 hours and less because we thought that a longer day-length might mask any temperature effect (as apparently it did in the experiments of Kendeigh, 1941). Also, day-lengths of less than 12 hours more nearly correspond to the natural day-lengths of winter, when birds normally are exposed to lower temperatures. Three different photoperiods were used to permit expression of the effect of length of day independently of the effect of temperature. We chose a test period of only six weeks again to prevent any masking of the effect of temperature—what was to be measured, essentially, was the rate of re-growth of the testes, and over a longer period of time birds exposed to the more adverse

experimental environment might have approached the plateau of the growth curve reached earlier by birds enjoying more favorable conditions.

Admittedly, it would have been immensely desirable to begin all test periods on or near the winter solstice, when natural day-length is shortest and when, presumably, the testes of any Junco taken in the field would be in an entirely inactive state. This was not done for the primary reason that we were unable to obtain birds in numbers sufficient to our purpose by that time. Bearing in mind, then, that by February, when some birds in each of the groups A through F were taken, spermatogenic activity had begun, with at least a considerable increase in the number of large Leydig interstitial cells, we held all birds of these groups for about eight weeks on a 10-hour photoperiod, expecting that on such a photoperiod and for such a time all birds would be reasonably "evened up." (We had the further consideration that by late spring the sun would have heated up the attic, where our light-tight compartments are installed, thus increasing the temperature contrast with the cold-room, but this now seems to have been an unnecessary precaution.) At any rate, groups G, H and especially J, which were put on test schedules on Dec. 21, provide some basis for comparison, and for judging the validity of our results.

We are indebted to our colleagues, Dr. Harry Smith and Mr. Edmund Gehan, of the Department of Biostatistics, for statistical analyses of testes volume.

Results

The day before the first three groups were started on a 12-hour photoperiod one bird of group A was found dead, and one also of group B, the deaths presumably due to injury occurring during transfer to clean cages. They provide some indication of the condition of the testes at this time, after about eight weeks on a 10-hour photoperiod (Feb. 16–Apr. 10). The combined volume of right and left testes of one was 2.5 mm.^{3}, the other 1.4 mm.^{3} Both were in stage III (Blanchard, 1941), not yet having reached the stage of division of spermatocytes.

12-hour photoperiod. Six weeks after the beginning of the test period on Apr. 11, the average paired testes volume in the birds of group A had increased approximately 50-fold, reaching 116.2 mm.^{3} (Table I). However, these birds, in warm cages, were far advanced in testicular development over the birds of group B, held in a relatively cold environment, the average volume of the paired testes in group A being approximately four times that of the testes in group B. They were similarly advanced over the birds of group C, which were warmed during the lighted portion of the day but exposed to the cold daily during the dark period. The differences in average volume are significant at the 0.1% level, both between A–B and A–C. The apparent difference between B and C is not statistically significant.

Fully formed spermatozoa were present in at least small numbers in each tubule of seven of the eight birds in group A, but only six of fifteen birds in groups B and C had reached that condition (Blanchard's late stage VI), thus 88% in A, only 40% in B and C. None of the testes from birds of groups B and C had tubules packed with sperm bundles, although four in group A had attained that stage (Blanchard's stage VII).

11-hour photoperiod. The difference between birds exposed to high and low temperatures when on an 11-hr. photoperiod (groups D and E, Table I) is less

marked, the difference in average testes volume being significant only between the 5%–10% levels. This low level of significance is largely due to the great variability shown by the birds of group D—in four of the nine, paired testes volume ranged from 18.2 mm³ to 52.3 mm³, while in another four it ranged from 2.9 mm³ to 5.4 mm³. In group E, however, the largest paired testes volume was 12.2 mm³; in six of the eight birds it ranged from 3.0 mm³ to 4.6 mm³. Of these two groups on an 11-hr. photoperiod the testes of only one bird had developed spermatozoa (Blanchard's late stage VI), this being the most advanced bird in group D. The

TABLE I

Summary of experimental conditions and resultant data

Group	n	Dates	Daily temp. °C.		Photo-period hrs.	Mean vol. pr. testes mm³	No. with sperm	Beyond stage III*
			Ave. max.	Ave. min.				
A	8	Feb. 16–Apr. 11	‡	‡	10			
		Apr. 11–May 24, '54	29.0	23.5	12	116.2	7	100%
B	7	Feb. 16–Apr. 11	‡	‡	10			
		Apr. 11–May 24, '54	8.6	4.0	12	26.3	2	100%
C	8	Feb. 16–Apr. 11	‡	‡	10			
		Apr. 11–May 24, '54	25.2	4.0	12	35.6	4	100%
D	9	Feb. 16–Apr. 9	26.3	20.6	10			
		Apr. 9–May 21, '55	29.9	24.9	11	16.9	1	100%
E	8	Feb. 16–Apr. 9	26.6	20.8	10			
		Apr. 9–May 21, '55	8.6	4.5	11	5.4	0	75%
F	7	Feb. 16–Apr. 9	26.8	20.6	10			
		Apr. 9–May 21, '55	29.3	24.5	10	5.2	0	30%
G	8	Dec. 21–Feb. 1, '55	26.3	19.5	11	1.4	0	0
H	7	Dec. 21–Feb. 1, '55	8.2	4.3	11	1.2	0	0
J	4	Dec. 21–Feb. 16	25.0	19.5	11			
	(+ 2 ♀♀)	Feb. 16–Apr. 9	25.9	20.6	11			
		Apr. 9–May 21, '55	28.9	24.5	11	14.9	1	100%

* Attainment of stage IV, beginning of spermatocyte division, coincides in white-crowned sparrows with the beginning of territorial activity in resident populations; in migratory populations it shortly precedes start of the spring migration (Blanchard, 1941).

‡ Temperatures were not recorded for groups A, B, and C during the period Feb. 16–Apr. 11, 1954; but these birds were in the same compartments as used for D, E and F in 1955 and temperatures were certainly in the general range 20°–25° C.

difference in average testes volume between B (12-hr., cold) and D (11-hr., warm) is not demonstrably significant; that between C (12-hr., warm day, cold night) and D is on the borderline of statistical significance.

When an 11-hr. photoperiod was similarly tested but beginning Dec. 21, advance in spermatogenesis was slight by the end of the test period on Feb. 1 (groups G and H, Table I). The testes of no one of these birds reached stage IV, the beginning of division of spermatocytes, although one in group G was very near to that point. Most were in stage III. The difference between G and H in testes volume

is not statistically significant, but it may be noted that it is the cold group H which shows the lowest average volume and that it is the warm group G in which the most advanced individual occurs. Measurements of maximum tubule diameter agree with calculation of testes volume—in the warm group G maximum tubule diameters averaged 75.8 μ, in the cold group H only 66.7 μ.

In another experiment (group J, Table I), four males were exposed to warm temperatures and to a day-length of 11 hours for five calendar months (Dec. 21–May 21). Two females were caged with them throughout this time, an arrangement which we expected would enhance the chances of the males developing larger testes, as has been demonstrated, on a longer day, for starlings (Burger, 1953). In only one Junco of this group, however, did the testes develop to a stage at which spermatozoa were present (45.8 mm^3, late stage VI). The next largest pair of testes had a volume of 6.9 mm^3 (stage V), the smallest only 2.3 mm^3 (early stage IV, *i.e.*, first appearance of spermatocyte division). The range of development was close to that in group D (largest 52.3 mm^3 in D, 45.8 mm^3 in J; 55% under 10.5 mm^3 in D, 75% under 7.0 mm^3 in J).

10-hour photoperiod. For practical reasons having to do with available space and available numbers of male Juncos, a 10-hr. photoperiod was not tested in the cold-room, but only in the warm temperatures of the attic (group F, Table I). None of the seven birds in this test developed spermatozoa, although the testes of one, by far the most advanced, reached a mature stage V, the tubules being packed with primary spermatocytes undergoing division (testes volume 22.6 mm^3). The testes of only one of the remainder showed any dividing spermatocytes (stage IV; testes volume 3.0 mm^3). Testes of five of the seven birds were still in stage III; in three of these, testes volume was less than 2.0 mm^3. The largest pair of testes exceeded the next largest by more than 500%. If this extreme high value is rejected from the statistical analysis, then the difference in the average volumes between group E (11-hr., cold) and group F (10-hr., warm) is significant at the 2%–5% level. That is, in most Juncos recrudescence of the testes proceeds at a measurably faster rate in the cold on an 11-hr. photoperiod than in warmth on a 10-hr. photoperiod.

Discussion

The above results seem clearly to demonstrate that if Juncos are subjected to low temperatures during the period of testicular recrudescence the rate of recrudescence will be retarded (or that, conversely, the rate will be accelerated in birds enjoying a warmer environment). This temperature effect is especially concise in groups A and B, as also in A and C; it is evident, too, in groups D and E. Comparison of groups B and C at least suggests that average daily temperature may be more important than minimum temperature, although the data are insufficient for a precise demonstration.

To this extent our experimental results confirm the conclusions drawn from field observations by Blanchard (1941) and by Marshall (1951) as to the effect of temperature on the annual recrudescence of the avian testis. But additionally they demonstrate, we believe, a decisive limitation on the favorable effects of warm temperature, the limiting factor being a favorable day-length, confirming then also the conclusions of Burger (1948). Two of seven of our birds on a 12-hr. photoperiod

in a continuously cold environment developed spermatozoa, as well as four of eight exposed to warm days and cold nights, but only one of nine in continuously warm surroundings on an 11-hr. photoperiod did so. Thus, rate of testicular recrudescence in Juncos under favorable temperature conditions on an 11-hr. photoperiod is certainly no faster, and is perhaps somewhat slower, than it is under poor conditions of temperature but with an additional hour of light (group D versus group B or C). In testes of six of eight birds kept in the cold-room on an 11-hr. photoperiod (group E) primary spermatocytes were undergoing division; on a 10-hr. photoperiod in a warm environment (group F) only two of seven attained this stage. In neither of these groups were spermatozoa developed. If we combine results on different day-lengths (groups A through F) we note that while thirteen of twenty-three birds on a 12-hr. photoperiod (56%) developed spermatozoa, only one of seventeen on an 11-hr. photoperiod (6%) and none of seven on a 10-hr. photoperiod (zero %) did so. Therefore, at these day-lengths and temperatures—both of which are reasonable when compared to conditions in nature—length of the photoperiod is shown clearly to be a more fundamental limiting factor in rate of testicular recrudescence than is temperature. Evidence that this conclusion is valid also for another passerine bird (*Zonotrichia leucophrys gambeli*) is apparent in the data of Farner and Mewaldt (1953), since their individuals kept at about 0° C. on a constant 15-hour day far exceeded, in testicular development, individuals kept in surroundings of 21° C. on natural day-lengths under 12 hours long. Kendeigh's (1941) conclusion that temperatures averaging 2° C. do *not* limit gonad development is not contradictory to our results with low temperatures; the seeming contradiction receives a logical interpretation in terms of day-length. That is, Kendeigh employed a long photoperiod (up to 15 hours), thus further augmenting the already stronger influence of length of day, and this augmentation overrode completely, or at least eventually, the retarding effect of low temperature.

If the validity of our results is questioned because of the several weeks through which we held birds on a 10-hr. photoperiod before beginning the test schedules, we may emphasize justifiably our experiment J, despite the small number of birds involved. These four male Juncos were placed on an 11-hr. photoperiod on the day of the winter solstice; although they were kept warm and had the company of two females, only one had developed spermatozoa even after five months. The testes of this one were next to the smallest of any of the fifteen which developed spermatozoa in these experiments and, judging from tubule size, were just out of the stage of predominance of spermatids. The next largest pair of testes in this group J was smaller than any but one of the twenty-three in our 12-hour groups. The range of development was entirely comparable to that in group D, which were on an 11-hr. photoperiod from Apr. 9 to May 21. All of this strongly suggests to us that on an 11-hr. photoperiod under otherwise favorable experimental conditions most Juncos cannot develop spermatozoa even after several months.

The relatively slight testicular development in those birds exposed to an 11-hr. photoperiod for six weeks beginning at the winter solstice (groups G, H) suggests that our later birds (groups A–F) were somehow conditioned to a more rapid, or greater, response as a result of their long experience of a 10-hr. photoperiod following natural day-lengths to Feb. 16. This is in agreement with the findings of Vaugien (1955) on young house sparrows (*Passer domesticus*). His data demon-

strate a progressive increase in testicular weights of sparrows exposed to continuous illumination for four weeks beginning at successive intervals from fall to spring. We would expect, then, if all experiments had been started on Dec. 21, a lesser degree of response in each group after six weeks of treatment. But since all the birds of our groups A to F had experienced essentially identical day-lengths prior to mid-April, the nature of the differences in results can be ascribed confidently to differences in the experimental environment during the ensuing six weeks.

We have not conclusively demonstrated the existence of a minimum or critical length of day, since at least some spermatogenic activity occurred even in our 10-hr. group. The possibility remains that on the shortest days there proceeds, albeit very slowly, at least the earliest stages of recrudescence, *i.e.*, development of functional interstitial cells and multiplication of spermatogonia. Our results suggest, however, that for Juncos days longer than 10 hours are necessary at least to pass beyond the first meiotic division, and it may well be that it is at this stage that length of day becomes the critical factor. An essentially similar conclusion is drawn by Miller (1955) with respect to golden-crowned sparrows (*Zonotrichia coronata*), from results of experiments in which birds were placed on a 10-hr. photoperiod beginning at the winter solstice and sampled at intervals during the following spring and summer. He interprets his data as demonstrating an innate tendency to recrudesce, more or less on schedule, but notes that, on a constant 10-hr. day, this innate process falls short of the level of completion of the maturation divisions, reaching a maximum of stage V with subsequent regression to a resting state.

Our experiments were not continued over as long a period as these of Miller. However, one of our Juncos on a 10-hr. photoperiod reached stage IV, and one which reached stage V approached the next stage, the appearance of spermatids. The relatively advanced state of the testes of this one bird may perhaps point to a further conclusion, when considered together with extreme cases in other groups. In each of five of the six groups A through F, one individual was considerably advanced over all others in its group; in the other group (C) two were so advanced. In group A, the largest pair of testes exceeded the next largest by 25%, in group B this value amounted to 60%, (in C to 10%), in D to 80%, in E to 54%, in F to 510% (and in J to 563%!). In group C the average of the two largest pairs of testes exceeds the third largest pair by 63%; the comparable value in A is 43.5%. That such variations are natural phenomena, and not bizarre experimental results, is evident in recorded field observations, for example, those of Blanchard (1941, p. 56 and Fig. 25) on white-crowned sparrows and of Kirschbaum and Ringoen (1936, Table I) on English sparrows. An explanation for these exceptional Juncos, which we believe to be an entirely plausible and reasonable one, lies in the prospect that individuals vary as to the threshold of response to external stimuli to testicular recrudescence, and that for a very few individuals this threshold is relatively low, occurring even perhaps on a 10-hr. photoperiod and manifesting itself at least under favorable temperature conditions after a long period of time.

Our experiments do not touch on the question as to the point in Junco physiology at which either day-length or temperature exerts its primary or immediate effect. As to temperature it has been suggested by Davis and Davis (1954) that increased thyroid activity induced by a cold environment interferes in some way

with testicular activity, possibly from an antagonism of the thyrotropic and gonadotropic functions of the pituitary. At any rate, our results with a 12-hr. photoperiod in cold surroundings show that low temperature alone is not a limiting factor, and such experiments as Kendeigh's indicate that on relatively long days the photoperiodic mechanism completely overrides the temperature mechanism. Also, there are many recorded cases of sperm development in males, and production of fertile eggs in females, in birds exposed to freezing temperatures on a long photoperiod (*cf.* Burger, 1949). We must conclude that day-length is in some way an integral part of the mechanism of testicular recrudescence, being either permissive or prohibitive of sperm development above or below approximately 11 hours in Juncos, and that temperature plays at best a modifying role.

Summary

1. The problem of the environmental control of the initiation of seasonal gonad development in birds was studied experimentally by giving attention to the factors of temperature and day-length at levels near those occurring in nature at the time of normal recrudescence.

2. Forty-seven male Juncos, collected at Chapel Hill, N. C., in January and February, were placed, in groups of seven to nine, on a 10-hr. photoperiod in warm cages from mid-February to mid-April. They were then placed for six weeks on photoperiods of either 12 hours or 11 hours, and in one case continued at 10 hours. One of the 12-hr. groups and one of the 11-hr. groups were transferred to a cold-room at 4°–8° C. for this period. Another 12-hr. group was placed in the cold-room for the dark period only, removed during the daily light period. One 12-hr. group, one 11-hr. group and the 10-hr. group were continued in the warm cages, where temperatures ranged from 24° to 29° C.

3. After six weeks, testes of birds in the 12-hr. warm group were about four times as large as testes of birds in either of the 12-hr. cold groups. Testes of birds in the 11-hr. warm group were nearly as large as those in the 12-hr. cold groups and were larger than those in the 11-hr. cold group. Testes of birds in the 11-hr. cold group were significantly larger than those in the 10-hr. warm group.

4. Thirteen of twenty-three birds on a 12-hr. photoperiod (56%) developed spermatozoa; one of seventeen on an 11-hr. photoperiod (6%) and none of seven on a 10-hr. photoperiod (zero %) did so.

5. Four additional males, in warm (20°–29° C.) cages with two females, were exposed to an 11-hr. photoperiod beginning at the winter solstice; after five months only one had developed spermatozoa. Range of testes development in this group was comparable to that in the 11-hr. warm group above.

6. In another experiment fifteen males were placed on an 11-hr. photoperiod beginning at the winter solstice; after six weeks none had reached spermatogenic stage IV. However, in the 10-hr. group above, one reached stage IV, one stage V.

7. From these data, and from consideration of the pertinent literature, it is concluded *a*) that low temperatures retard the rate of testicular recrudescence in Juncos, higher temperatures accelerating this rate; but *b*) that day-length is in some way an integral part of the mechanism of recrudescence, being either permissive or prohibitive of sperm development above or below approximately 11 hours,

temperature having here only a modifying role; and *c*) that although the existence of a critical, minimum length of day is not conclusively demonstrated, it is not contra-indicated, at least as regards the critical stages of completion of maturation divisions.

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AN ENDOCRINE BASIS FOR THE SEXUAL DIFFERENCE IN MELANIN DISPERSION IN UCA PUGILATOR[1]

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The degree of dispersion of the pigment in the melanophores of fiddler crabs, *Uca* spp., is influenced by a variety of factors. Carlson (1935) demonstrated that blanching resulting from melanin concentration occurred in *Uca pugilator* when the eyestalks were removed. Injection of extracts of the eyestalks caused the *Uca* to darken through dispersion of the pigment. Sandeen (1950) showed that the central nervous organs produced a factor that caused melanin dispersion. Fingerman (1956a) found a blood-borne hormone that concentrated the melanin in the chromatophores of *Uca pugilator*.

Light, temperature, and background also influence the state of the pigment (Brown and Sandeen, 1948). The dispersion of the melanin increased with increase in the light intensity. The black pigment tended to concentrate with increase in temperature. An albedo response was also present. The black pigment tended to disperse on a black background and concentrate on a white background.

Daily and tidal rhythms also determine the dispersion of the melanin. Abramowitz (1937) first described the daily rhythm. The *Uca* were dark by day and light by night. Brown and Webb (1949) and Webb (1950) elucidated the mechanism involved in shifting the phases of this rhythm.

Superimposed upon the daily rhythm is a tidal rhythm (Brown, Fingerman, Sandeen and Webb, 1953; Fingerman, 1956b). This tidal rhythm leads to an increased degree of dispersion of the melanin approximately two hours later than the actual time of occurrence of the tides which are spaced 12.4 hours apart. In regions with semidiurnal tides this augmentation occurred only after the low tide, but in regions of diurnal tides this augmentation occurred after both high and low tides.

Examination of a population of fiddler crabs, *Uca pugilator*, revealed that the females were darker than the males. The following investigation was undertaken to analyze the difference in coloration between the males and females.

Materials and Methods

The specimens of *Uca pugilator* used in these experiments were collected near the Gulf Coast Research Laboratory at Ocean Springs, Mississippi, in August, 1955. Several thousand adults were collected and transported on the same day to an air conditioned laboratory in New Orleans, Louisiana, where the room temperature was 24° C. The *Uca* were retained in stainless steel aquaria containing sea water changed every second day. They were kept in constant darkness from the evening of the day they were collected until they were used in the experiments.

[1] This investigation was supported by Grant No. B-838 from the National Institutes of Health.

To determine the degree of dispersion of the pigment in the melanophores the method of Hogben and Slome (1931) was used. Each *Uca* was placed on the stage of a stereoscopic dissecting microscope and the average stage of the chromatophores on the anteroventral aspect of a walking leg was determined. According to this system, stage 1 represents maximum pigment concentration, stage 5 maximum dispersion, and stages 2, 3, and 4 the intermediate stages.

Experiments and Results

Sexual difference in the degree of pigment dispersion

Fifty males and 50 females were selected from the stock supply at 10:00 A.M. for use in this experiment. Twenty-five of these females were then placed into each of two white enameled pans. Likewise 25 of the males were placed into each of two white enameled pans. These pans were then placed under a constant illumination of 20 ft. c. intensity. From 4:00 P.M. through 8:00 P.M. the average chromatophore stage of 20 *Uca* from each pan was determined at hourly intervals. After the 8:00 P.M. determination the large chela was removed by autotomy from the 25 males in one of the pans. Male *Uca* have one large chela ("fiddle") and one small chela. Female *Uca* have two small, equal-sized chelae. The small chela on the left side of all the females in one pan was removed at the same time. The average chromatophore stage of 20 of the *Uca* in each of the four pans was then determined at hourly intervals from 8:00 A.M. through noon on the following two days.

This experiment was repeated once. The results have been presented in Figure 1 where each point represents the average of 40 *Uca*. As is evident from section A of the first figure, the normal females were darker than the normal males. The females which had lost one chela became slightly darker than the normal females (Fig. 1; B, C). The normal males remained lighter than the normal females. However, the males which had lost the large chela were darker than both the normal males and normal females but not quite as dark as the females which had lost one chela. The average chromatophore indices for the males without the large chela lay between the values determined for the two groups of females. Apparently, the sexual difference in coloration was due to the presence of the large chela on the males.

To illustrate better the effect of removal of the chelae upon the coloration of both sexes, the data were treated in the following fashion. The five average chromatophore indices obtained on each of the three days for each group of *Uca* were summed. This procedure produced 12 sums. Five was then subtracted from each sum because if there had been no pigment dispersion the sum would have been five. These values were a measure of the chromatophore activities for each day. The average activity for the two groups of normal males was 3.1 activity units and for the normal females 9.4 activity units, a three-fold difference (Fig. 1A).

The activities determined for each of the four groups of *Uca* on the last two days of the observations (Fig. 1, B, C) were summed. The sum of the activities of the normal males was then divided into the sum obtained with the males from which the large chela had been removed. The ratio was 2.8. In like manner, the ratio for the normal females to the females with a chela removed was found to be 1.5, almost a two-fold difference between the males and females.

In order to explain the results presented above the volumes of the large chela and of the body minus the large chela of 10 male *Uca* were determined and the average taken. The large chela of a male had a volume of 1.3 ml., the body and the nine remaining appendages had a volume of 1.5 ml. The results for 10 females showed that the body volume was 1.6 ml. and the volume of one of the small chelae was 0.04 ml.

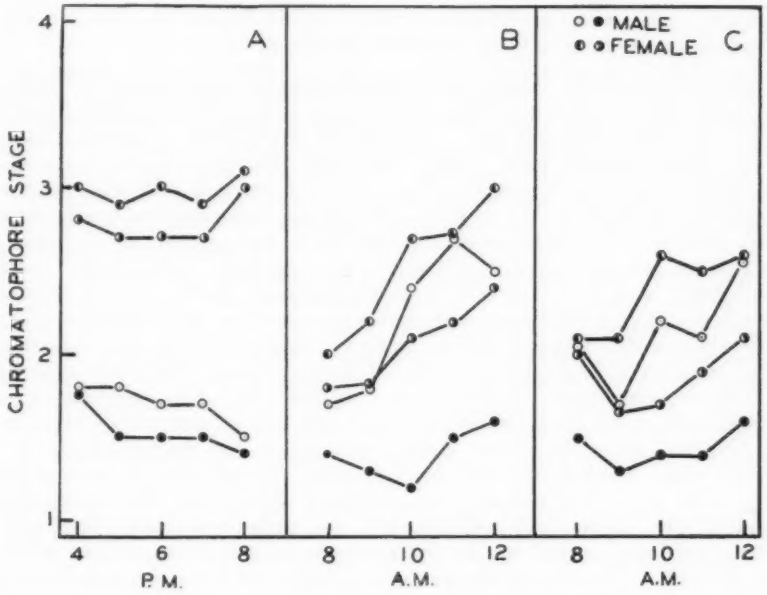

FIGURE 1. A comparison of the degree of pigment dispersion in males and females. In A the males and females were intact, normal *Uca*. In B and C 50 per cent of the males had lost the large chela and 50 per cent of the females had lost one chela.

- ● Males, normal
- ○ Males, large chela missing in B and C
- ◑ Females, normal
- ◐ Females, one chela missing in B and C

Effect of serial removal of appendages

At noon 25 *Uca* were placed into each of nine white enameled pans. Of the 25 *Uca* in each pan approximately 50 per cent were males and 50 per cent of the males had a large chela on the left side. Both sexes were used in this experiment in order to determine the amount of variation that might be due to loss of appendages in experiments where sex is not taken into account. The appendages were arbitrarily numbered consecutively from one to 10 beginning with the chela on the left side. The chela on the right side was appendage number six.

The *Uca* in the first pan were kept intact. The first appendage was removed from the *Uca* in the second pan, the first and second appendages were removed from the *Uca* in the third pan, and so on until the *Uca* in the ninth pan had two appendages remaining. The nine pans were then placed under an illumination of 40 ft. c. intensity. At hourly intervals from 8:00 A.M. through noon on the next two days the average chromatophore stage of 20 of the *Uca* in each pan was determined. The average of the 10 values obtained for each pan was then calculated.

This experiment was repeated once. The only change in the procedure was that the pans were placed under an illumination of 20 ft. c. instead of 40 ft. c. This change had no apparent effect.

The results of both experiments were averaged and have been presented in Figure 2 where each point represents the average of 40 *Uca*. In general, the

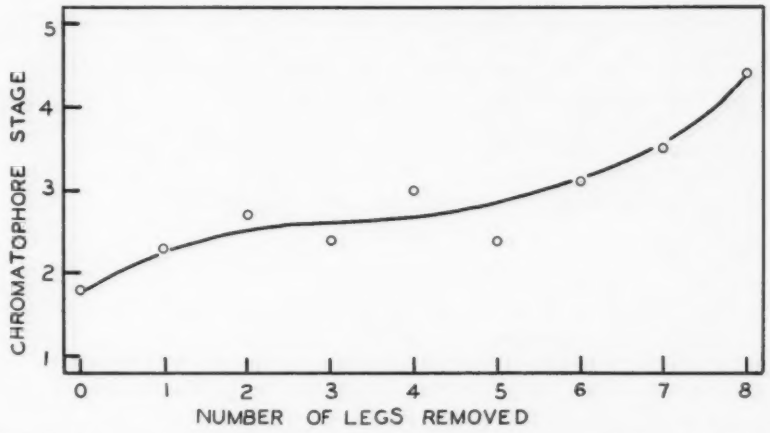

FIGURE 2. A comparison of the degree of melanin dispersion in normal *Uca* with *Uca* from which one to eight appendages had been removed.

greater the number of appendages which had been removed the darker were the *Uca*. The curve obtained was sigmoid with the greatest increase in coloration having occurred when seven and eight appendages had been removed.

To assure that the order in which the appendages had been removed did not effect the results, five appendages were removed from 25 *Uca*, the first three appendages from the left side and the first two from the right side, with no apparent difference in the results. Eight appendages, the first four from both sides, were also removed from 25 *Uca*. The average chromatophore stages of these two groups of *Uca* were similar to the results presented in Figure 2. The order in which the appendages had been removed had no effect upon the results.

DISCUSSION

These experiments have shown that female *Uca pugilator* were darker than the males. The results of the experiments which involved removal of the large male

chela showed that this difference in coloration was not a fundamental sexual difference but could be explained merely on the basis of hormonal titers in the blood. This sexual difference in body coloration was due only to the fact that the males with a large chela have a greater circulatory volume than the females. The same quantity of darkening hormone was secreted by the eyestalks and central nervous organs of the male and female *Uca*, but this hormone was diluted to a greater extent in the males which have a greater circulatory volume. Removal of the large chela from the males equalized the difference in the circulatory volume and the males then darkened to the same extent as the females because the hormone was then more concentrated in the blood of the males minus the large chela.

On the basis of the chela and body volumes, the hormonal titer in the blood of males can be assumed to be nearly doubled by removal of the large chela whereas removal of the chela from a female produced a minor volume change. This conclusion, of course, depends upon the assumption that the circulatory volume per unit volume of *Uca* was the same in the body and the large chela.

The second series of experiments also supported this hypothesis. The observation was made that the greater the number of appendages that had been removed, the darker were the *Uca*. This result can also be explained on the basis of a decreased circulatory volume which led to a concentration of the darkening hormone in the blood. An interesting observation is that the *Uca* did not compensate for the loss of the appendages by secreting less darkening hormone.

Summary and Conclusions

1. The pigment in the melanophores of female specimens of the fiddler crab, *Uca pugilator*, was more dispersed than the pigment in the males. This difference in pigment dispersion caused the females to appear darker than the males.

2. Removal of the large chela from the male resulted in approximately equal coloration of these males and normal females.

3. This apparent sexual difference in coloration was due to greater dilution of the darkening hormone in the blood of male *Uca* than in female *Uca* because of the greater total volume of the males and was not due to a fundamental sexual difference in the amount of darkening hormone secreted.

4. The greater the number of appendages removed, the darker were the *Uca*. These results have also been explained on the basis of the decrease of circulatory volume in which the hormone had become diluted.

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RESPONSES OF THE WHITE CHROMATOPHORES OF TWO SPECIES OF PRAWNS (PALAEMONETES) TO LIGHT AND TEMPERATURE[1]

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Light and temperature are the major environmental factors governing pigment migration in the chromatophore systems of animals. Light responses have been separated into two categories: (1) responses to total illumination, also known as primary responses, and (2) responses to background, also known as secondary responses. Primary responses are not operative through the eyes whereas secondary responses involve a reflex operating through the eyes. Dispersion of chromatophoric pigments with increased illumination is the typical primary response. Secondary responses typically result in dispersion of dark pigments on a black background and concentration on a white background. White chromatophores usually respond to background in a manner opposite to black pigment. Primary and secondary responses have been demonstrated in several species of crustaceans; among them are *Hippolyte varians,* a shrimp (Keeble and Gamble, 1900; Kleinholz and Welsh, 1937); *Leander serratus,* a prawn (Stephenson, 1934); *Ligia oceanica,* an isopod (H. Smith, 1938); *Uca pugilator,* a fiddler crab (Brown and Sandeen, 1948); and *Callinectes sapidus,* the blue crab (Fingerman, unpublished data).

Background adaptation (secondary response) depends upon the ratio of the light intensity emanating from the light source (incident light) to the intensity of the light reflected from the background. Both incident and reflected light intensities are measured at the surface of the eye. Incident light normally impinges upon the dorsal portion of the compound eye and reflected light upon the ventral portion. The ratio of incident to reflected light intensity is referred to as the albedo.

In 1938, H. Smith demonstrated that background responses of the isopod *Ligia oceanica* were not due simply to the albedo but were complicated by a functional differentiation of the retinal cells or neurons associated with them. By means of experiments in which various portions of the eye were either covered with an opaque material or differentially stimulated by adjustment of the background, he was able to demonstrate that stimulation of the dorsal portion of the eye caused pigment dispersion. Stimulation of the lateroventral portion of the eye caused pigment concentration which resulted in body lightening.

Temperature responses of chromatophores have not been as uniform as light responses. With high temperature, pigments disperse in some species, *e.g.,* the shrimp *Macrobrachium acanthurus* as shown by D. C. Smith (1930) and concentrate in others, *e.g.,* the fiddler crab *Uca pugilator* as shown by Brown and Sandeen (1948).

[1] This investigation was supported by Grant No. B-838 from the National Institutes of Health.

Chromatophores of the prawn *Palaemonetes vulgaris* have been the subject of several investigations. Brown (1935a, 1935b) demonstrated that the white chromatophoric pigment of this species disperses broadly when the prawn is on a white background and concentrates strongly when the prawn is on a black background. The white pigment of eyestalkless individuals was found in all states from fully concentrated to fully dispersed with no apparent dependence upon background or light intensity (Brown, 1935a). Hanström (1937) postulated that the white chromatophore of *Palaemonetes vulgaris* is an "independent effector." His experiments suggested that the primary determinant of the state of the pigment was total illumination which caused white pigment dispersion in bright light and concentration in dim light. Responses to total illumination were independent of the nature of the background.

Concentration and dispersion of white pigment of prawns on black and white backgrounds, respectively, are not necessarily due to a specific albedo response. Concentration and dispersion of pigments, especially retinal pigments, in some species may be governed solely by differences in the amount of light reflected from the background with no dependence upon the intensity of the incident light (Sandeen and Brown, 1952; Fingerman, unpublished data).

Neither the physiology of the chromatophores of the prawns *Palaemonetes pugio* and *Palaemonetes paladosus* nor the responses to temperature of the white chromatophores in the genus *Palaemonetes* have been determined previously. The study described herein was undertaken to investigate the white chromatophore responses of two species of prawns, *Palaemonetes pugio* and *Palaemonetes paladosus*, to light and temperature in order to determine the influence of environmental factors upon the white pigment.

Materials and Methods

Specimens of the prawns *Palaemonetes pugio* and *Palaemonetes paladosus* collected near Point Aux Herbes, 15 miles northeast of New Orleans, Lake Pontchartrain, Louisiana, were used in the experiments described herein. Prawns were selected for use without regard to sex or size from stock aquaria which contained diluted sea water at a temperature of 22–24° C. When used in experiments, prawns were placed in pans with a bottom diameter of 14 cm. Each pan contained sufficient water, usually one inch deep, to cover the prawns.

The white chromatophores in the portion of the carapace dorsal to the heart were chosen for observation. Approximately 25 per cent of the individuals of both species did not have white chromatophores dorsal to the heart and were discarded. Males formed a large majority of the discarded specimens.

The white chromatophores were staged with the aid of a stereoscopic dissecting microscope according to the system of Hogben and Slome (1931). Stage 1 represents maximum pigment concentration, stage 5 maximum dispersion, and stages 2, 3, and 4 the intermediate conditions.

Experiments and Results

Responses of white chromatophores to background

Twenty specimens of each species were selected from the stock aquaria and divided into groups of 10. One lot of each species was placed into black enameled

pans and the remaining specimens were placed into white enameled pans. The four pans were then placed under an illumination of 40 ft. c. light intensity for 120 minutes after which time the average chromatophore stage of the prawns in each pan was determined. Light intensities were measured by means of a General Electric Photometer. The *Palaemonetes* on the black and white backgrounds were then exchanged so that the prawns which had been originally on a black background were transferred to a white background and vice versa. Average chromatophore stages for the prawns in each pan were then determined 15 and 30 minutes after the backgrounds had been exchanged.

The experiment was repeated once and the results averaged to prepare Figure 1 in which each point represents the average of 20 individuals. As is evident from the figure, the white pigment of both species concentrated when prawns were placed

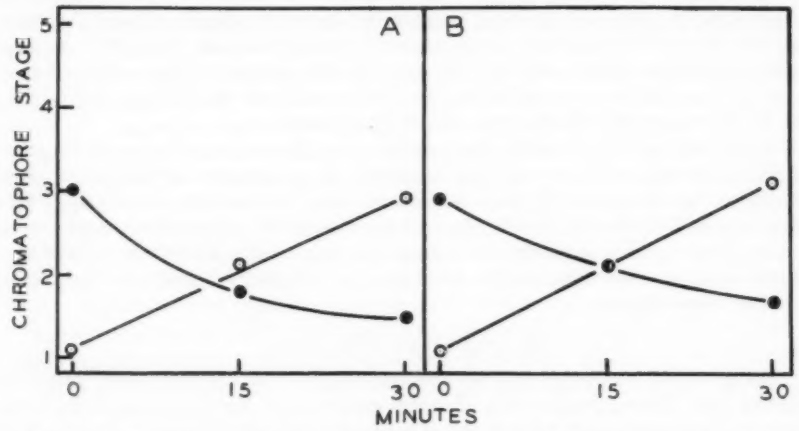

FIGURE 1. Responses of the white chromatophores of *Palaemonetes pugio* (A) and *Palaemonetes paladosus* (B) to background changes. Circles, black to white; dots, white to black.

on a black background (dots) and dispersed when prawns were placed on a white background (circles). No species difference in rate of white pigment dispersion or concentration was evident. The white pigment of neither species was maximally dispersed on a white background.

Responses of white chromatophores to illumination

One hundred prawns of each species were selected from the stocks and separated into 10 groups. Five lots of each species were placed in white enameled pans and the remaining groups were placed in black enameled pans. One white and one black pan for each species were then placed under one of the illuminations in the light gradient of 1, 10, 40, 120, and 2000 ft. c. intensity. The latter intensity was obtained with sunlight, the remaining intensities in the light gradient were obtained by placing the pans at appropriate distances from the light sources available in the

laboratory. The water in the pans placed in sunlight was changed at frequent intervals to prevent heating of the prawns. After 120 minutes of exposure the average chromatophore index of the prawns in each pan was determined.

The experiment was repeated once and the averaged results are presented in Figure 2 in which the average chromatophore index is plotted against the logarithm of the incident illumination. Each point represents the average of 20 individuals.

The white pigment of both species was more dispersed in prawns on a white background than on a black background at all intensities of illumination. Dispersion of the white pigment of both species of prawn on the black as well as the white background increased with increasing illumination although no significant dispersion of the white pigment occurred in prawns on a black background at illuminations below 120 ft. c. light intensity.

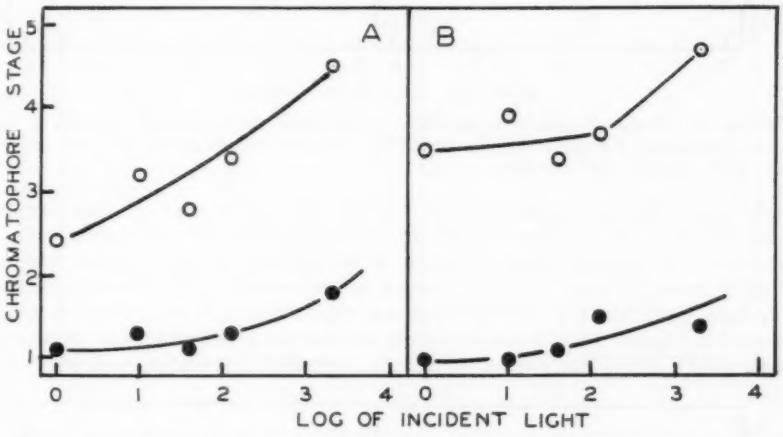

FIGURE 2. Relationship between dispersion of the white chromatophoric pigment of *Palaemonetes pugio* (A) and *Palaemonetes paladosus* (B) and the logarithm of the incident light intensity. Dots, black background; circles, white background.

A species difference was evident from the response of the chromatophores of prawns on a white background. The white chromatophores of *Palaemonetes paladosus* showed no response to illumination between the light intensities of one and 120 ft. c. whereas the white pigment of *Palaemonetes pugio* gradually became more dispersed as the intensity of illumination increased from one to 120 ft. c. In addition, the white pigment of *Palaemonetes paladosus* was more dispersed than the white pigment of *Palaemonetes pugio* at all intensities of illumination.

Additional experiments were performed in which prawns whose eyestalks had been removed 24 hours previously were exposed on a white background to the same intensities of illumination and for the same period of time used with the normal prawns. The white pigment of eyestalkless prawns was usually neither completely dispersed nor completely concentrated and frequently could be found in all stages of dispersion in one prawn as was observed in *Palaemonetes vulgaris* by Brown (1935a).

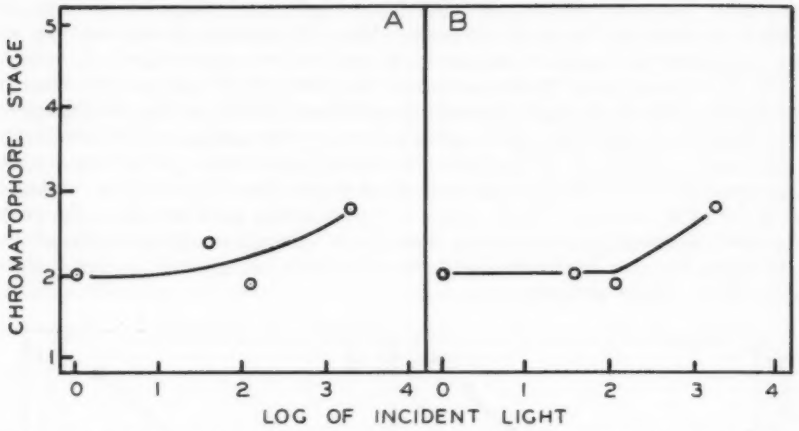

FIGURE 3. Relationship between dispersion of the white chromatophoric pigment of eyestalkless *Palaemonetes pugio* (A) and eyestalkless *Palaemonetes paladosus* (B) and the logarithm of the incident light intensity.

The experiment with eyestalkless prawns also was repeated once. The averaged results are presented in Figure 3 where each point represents the average of 20 individuals. A primary response of the white pigment of both species was evidenced by increased dispersion with increase in the intensity of incident illumination.

In view of the fact that the white pigment shows a primary response, the results presented in Figures 1 and 2 may be explained in terms of differences in the amount of light reflected from the black and white backgrounds that impinges directly upon

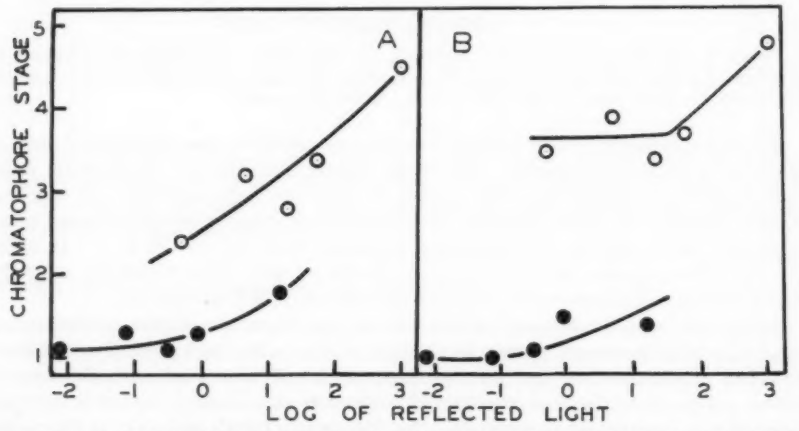

FIGURE 4. Relationship between dispersion of the white chromatophoric pigment of *Palaemonetes pugio* (A) and *Palaemonetes paladosus* (B) and the logarithm of the reflected light intensity.

the white chromatophores, rather than as an albedo or background response which is mediated by the eyes. Measurements by means of the General Electric Photometer showed the white backgrounds used in the experiments reflected 50 per cent of the incident light and the black background reflected 0.77 per cent of the incident light, 1/65 as much light as was reflected from the white background.

The data of Figure 1 were, therefore, re-plotted in terms of the intensity of the light reflected from the black and white backgrounds in order to assist in determining if the albedo is involved in the responses of the white chromatophores on black and white backgrounds (Fig. 4). As is evident from the figure the white pigment of both species responded directly to illumination because dispersion of the white pigment of both species increased with increase of the reflected light intensity. In addition, a specific albedo response was evident because the data ob-

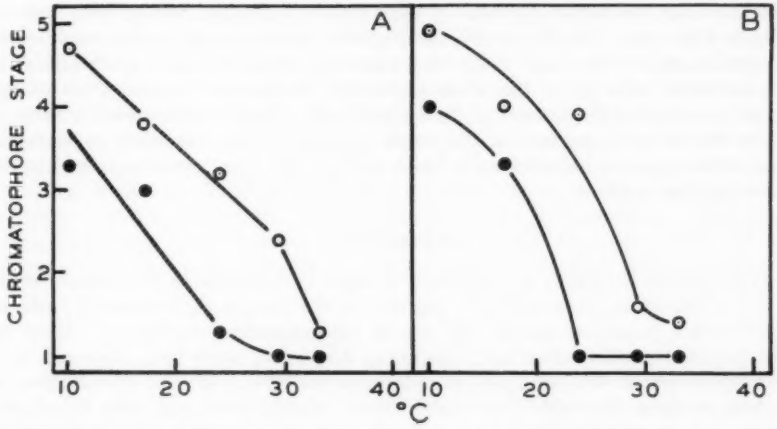

FIGURE 5. Relationship between temperature and white pigment dispersion in *Palaemonetes pugio* (A) and *Palaemonetes paladosus* (B). Dots, black background; circles, white background.

tained on the black and white backgrounds did not overlap although the incident light intensity had increased 2000-fold as compared with only a 65-fold increase in the reflected light intensity.

As is evident from Figures 1, 2, 3, and 4 dispersion of the white pigment of both species of *Palaemonetes* is due in part to summation of primary and secondary responses. The response to total illumination is an increased dispersion with increased illumination. The background response results in greater dispersion of the white pigment of prawns on a white background than of prawns on a black background.

Temperature responses of white chromatophores

Ten groups of 10 prawns of each species were selected from the stocks. Five lots of each species were placed in white enameled pans and a similar number in black enameled pans. Sufficient water to cover the prawns was then added and

the pans were placed in water baths. The temperatures of the five water baths were maintained at 10, 17, 24, 29, and 33° C., respectively. The incident illumination was 20 ft. c. light intensity. In each water bath were two black and two white pans containing individuals of each species. Each lot of prawns was kept in the bath for 120 minutes after the water in the pans had attained the temperature of the water bath. The average chromatophore index of the 10 prawns in each pan was then determined. The results are presented in Figure 5.

As is evident from the figure, at all temperatures the white pigment was more dispersed on a white background than on a black background. With increase of temperature, the white pigment of individuals of both species became more concentrated on both black and white backgrounds. No difference between the temperature responses of the white pigment of the two species was evident. The white pigment of individuals on a black background became fully concentrated at temperatures at which the white pigment of specimens on a white background was still partially dispersed. The four curves in Figure 5 are as parallel as one could expect in experiments of this sort. One may conclude from this parallel response that the qualitative behavior of the white pigment in response to temperature changes was independent of the nature of the background. Black backgrounds merely decreased the initial dispersion of the white pigment so that complete concentration of the white pigment occurred on a black background at a lower temperature than on a white background.

Discussion

Temperature is equally as important as light in determining the amount of dispersion of the white chromatophoric pigment of the prawns *Palaemonetes pugio* and *Palaemonetes paladosus* under any set of environmental conditions. Maximum white pigment concentration and maximum dispersion were both observed in the experiments which were designed to determine the effects of total illumination and temperature upon the white chromatophores. Background and total illumination both caused maximum white pigment dispersion and concentration in *Palaemonetes vulgaris* (Brown, 1935b; Hanström, 1937). *Palaemonetes paladosus* and *Palaemonetes pugio* are obviously exceptions to the general principle that light is more important than temperature in determining the state of the chromatophore system (Brown, 1950).

A thermoregulatory function of the white pigment does not seem plausible from the data presented herein. If the temperature response of the white chromatophores were thermoregulatory, then with increase in temperature, the white pigment should disperse in order to increase the area of the body surface which is able to reflect light and heat efficiently.

In contrast to the white chromatophores of *Palaemonetes* the melanophores of frogs appear to be thermoregulatory. More light is reflected from the skin of a frog whose black pigment is concentrated because of adaptation to a white background than from the skin of a frog whose melanin is dispersed due to adaptation to a black background (Deanin and Steggerda, 1948). These investigators have also shown that the skin of the frog reflects more red light than violet light, which lends further support to the thermoregulatory function of the frog melanophores because red light has a higher heating ability than violet light.

The white chromatophoric responses of the two species of prawns to temperature are antagonistic to their responses to illumination. With increased temperature the white pigment concentrated, whereas the pigment dispersed with increased total illumination. These antagonistic responses tend to maintain a steady state of the white pigment. Since heat and bright light are concomitant in nature, *e.g.*, sunshine, the tendency of the white pigment to disperse in bright light is counteracted by the tendency of the pigment to concentrate because of the associated heat.

In Lake Pontchartrain, where the *Palaemonetes* were collected, temperature changes probably affect the prawns more than changes in illumination. *Palaemonetes* in their habitat are not normally exposed to bright illumination but the water shows wide variation in temperature.

The white pigment of the fiddler crab *Uca pugilator* responds to temperature differently than the white pigment of *Palaemonetes pugio* and *Palaemonetes paladosus*. The white pigment in *Uca pugilator* tends to disperse as the temperature is increased above, or decreased below about 20° C. (Brown and Sandeen, 1948).

The white chromatophores appear to function as "independent effectors" in response to both total illumination and temperature. Evidence presented herein shows that white pigment concentration and dispersion in *Palaemonetes pugio* and *Palaemonetes paladosus* are due to summation of (1) an albedo response resulting in greater dispersion on a white background, (2) a response to total illumination resulting in greater dispersion in bright illumination, and (3) a response to temperature resulting in greater concentration at higher temperatures. Responses to temperature and total illumination appear to be more important than the albedo responses which are superimposed upon the primary response to total illumination.

Summary and Conclusions

1. Responses to light and temperature of the white chromatophores of two species of prawns, *Palaemonetes pugio* and *Palaemonetes paladosus*, have been investigated.

2. The white pigment is more dispersed in prawns on a white background than in prawns on a black background at all intensities of illumination, due to a specific background (albedo) response.

3. A response to total illumination is evident. The dispersion of the white pigment increased with increase in total illumination.

4. The white pigment became more concentrated with increased temperature in prawns on both black and white backgrounds.

5. The tendency of the white pigment to disperse in bright light is normally antagonized by the tendency of the pigment to concentrate with increased temperature. Since heat and bright light are usually concomitant in nature, as in sunlight, the antagonistic response to light and temperature has been interpreted as a mechanism to maintain a steady state of the white chromatophores.

6. At all intensities of illumination the white pigment of *Palaemonetes paladosus* was more dispersed on a white background than was the white pigment of *Palaemonetes pugio*. In distinction to *Palaemonetes pugio* the white pigment of *Palaemonetes paladosus* on a white background showed no response to illumination between one and 120 ft. c. light intensity.

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ANTIMITOTIC SUBSTANCES FROM THE OVARIES OF VERTEBRATES[1]

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More and more evidence has been accumulated to show that the protoplasmic colloid is in a state of dynamic equilibrium between the factors which tend to cause it to gel or clot and those which tend to prevent or reverse this gelation. For a review of much of this evidence, see Heilbrunn (1956).

Substances which are potent in preventing protoplasmic clotting can be obtained from the ovaries of various animals (Heilbrunn, Chaet, Dunn and Wilson, 1954; Heilbrunn, Wilson and Harding, 1951). The fact that the ovaries of some fishes contain such substances has already been noted. In this paper, additional data will be presented, not only for the ovaries of fishes, but also for the ovaries of other vertebrate animals. These substances which prevent protoplasmic clotting also prevent the mitotic gelation which is a necessary precursor of the mitotic spindle; hence such substances have an antimitotic action.

The methods used and the plan of attack were much the same as in the earlier work. Table I shows the results obtained in the summer of 1953. Ovaries from 8 species of fishes all contained substances which had a strong antimitotic action on the eggs of the worm *Chaetopterus pergamentaceus*. The extracts were prepared by homogenizing the ovaries in sea water which had been made acid by the addition of HCl in sufficient amount to break down the buffers of the sea water. Following homogenization, within a few minutes the extracts were centrifuged (at 16,000 g) until most of the solid material was removed, and the supernatant fluid was then brought to a pH of approximately 8, that is to say, to the pH of sea water. In the case of the ovaries of *Sphaeroides maculatus*, the extracts were made in ordinary sea water rather than in acid sea water.

Two minutes after they had been inseminated, Chaetopterus eggs were introduced into the ovarian extracts, used either full strength, or diluted one-half or one-fourth with sea water. The eggs were kept at a temperature of 21° C. In all cases, the cleavage, if it occurred at all in the extracts, was delayed. Cleavage counts were made after a time interval long enough to insure that the percentage of cleavage was as high as it ever would be. In some instances, a cleavage furrow would start to form, but then it would disappear and the egg would become spherical again. Viscosity determinations were made 30–40 minutes after insemination. At this time, normal eggs show increased protoplasmic viscosity. Earlier work (Heilbrunn and Wilson, 1948) had shown that on our arbitrary scale, the viscosity of the protoplasm rises from 7 to 14. This means that it takes 14 seconds, for a cen-

[1]This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service.

trifugal force of approximately 2200 g to move the granules of the protoplasm a sufficient distance so that the egg appears to be divided into zones. At this time, then, there is a gelation of some part of the protoplasm; this we call the mitotic gelation. In the control eggs such a gelation was always present. On the other hand, the protoplasm of the eggs exposed to the ovarian extracts always retained its original fluidity, and in some cases at least became even more fluid. This is indicated by the viscosity values shown in the table (Table I). In making these viscosity determinations, it was often not possible to make enough tests to be cer-

TABLE I

Effects of extracts of fish ovaries on cleavage of Chaetopterus eggs

Species	Extract no.	Grams ovaries	Ml. sea water	Viscosity 30–40 min. after insem.	% cleavage	Control % cleavage
Sphaeroides maculatus (Bloch and Schneider) puffer	1	72	100	6	0	100
Fundulus heteroclitus (Linnaeus) mummichug	6	10	25	4	0	97
	6	10	50	6	50	97
	6	10	100		46	97
	32	2	10	8	39	98
Opsanus tau (Linnaeus) toadfish	7	30	60	6 or less	0	99
	7	30	120	6 or less	0	99
Tautogolabrus adspersus (Walbaum) cunner	8	3	15	6	9	100
	33	1	9	8 or less	42	98
Tautoga onitis (Linnaeus) tautog	20	12	24	6	46	94
	29	15	30	8	15	100
Stenotomus versicolor (Mitchill) scup	22	2	10	8 or less	35	98
	22	2	20		37	98
	22	2	40		75	98
	31	2	10	6 or less	22	97
Anguilla rostrata (LeSueur) eel	35	6	30	8	49	89
Lophius americanus (Cuvier and Valenciennes) goosefish	4	100	100		0	93
	4	100	200		27	93

tain of the exact value for the viscosity, but in every case it was clear that the viscosity of the treated eggs was much less than that of the control (untreated) eggs centrifuged at the same time. These control eggs showed no indication of zones when they were centrifuged at the same force and for the same time as the treated eggs; whereas the eggs treated with a sufficient concentration of the ovarian extract, showed zones clearly. These treated eggs regularly gave viscosity values of 8 or less.

In the summer of 1954, the work on fish ovaries was continued. We made extracts from the ovaries of 7 other species of fish, and we also made new extracts from the ovaries of the toadfish (*Opsanus tau* L.). In the 1954 work, for each

gram of ovary, two ml. of sea water were used to obtain an extract. In the tests, the extracts were used full strength, half strength (that is to say, diluted with an equal volume of sea water), one-fourth strength, and one-eighth strength. In all cases the pH of the extract was adjusted so that it was approximately the same as that of sea water. The results of these 1954 experiments with fish ovaries are shown in Table II. Because of the fact that in most cases extracts weaker than half strength had but little effect, results obtained with these dilute extracts are not included.

Actually, our extracts always contain a mixture of substances which inhibit clotting and substances which promote clotting. If the extracts are allowed to age, the anti-clotting substances seem to lose their potency before the clotting substances do. Hence in extracts kept for a day or two in the icebox, the anti-clotting effect is reduced. Moreover, in one experiment done on the ovaries of fish (mackerel)

TABLE II

Additional data on the effect of extracts of fish ovaries on the cleavage of Chaetopterus eggs. The figures show percentage of eggs cleaving

Species	Full strength extract	Half-strength extract	Control
Brevoortia tyrannus (Latrobe) menhaden unripe ovary	10	17	100
Same—ripe ovary	1	4	100
Raja erinacea (Mitchill) skate—unripe ovary	0	48	97
Same—ripe ovary	0	80	97
Poronotus triachanthus (Peck) butterfish	96*	90	100
Opsanus tau (Linnaeus) toadfish	0	25	99
Centropristis striatus (Linnaeus) sea bass	10	81	99
Prionotus carolinus (Linnaeus) sea robin	76	86	97
Pseudopleuronectes americanus (Walbaum) winter flounder	11	96	100

* Cleavage was delayed for well over 6 minutes.

bought in a fish market, we found that the extracts caused clotting rather than liquefaction of the protoplasm. In every case, the ovarian extracts prepared from freshly caught fish kept the protoplasm fluid and prevented the mitotic gelation. This was determined by tests of viscosity made 30–40 minutes after insemination.

Table III shows the effect of extracts of ovaries from vertebrates other than fishes. These extracts also have a strong antimitotic effect, and in general they also prevent the mitotic gelation. Only one experiment with cow ovaries is listed. As a matter of fact, in attempting to discover how to make more potent extracts, we did many experiments with extracts from cow ovaries. The results obtained will be reported later. Suffice to say here that we always found sea water extracts of cow ovaries to exert a strong antimitotic action. However, in making these extracts we found it advisable to mince the ovaries instead of homogenizing them. Also it was not necessary to acidify the sea water in order to obtain a satisfactory extract.

Our results show that the ovaries of various vertebrate animals all contain antimitotic substances. At the present time, we are trying to obtain these substances

TABLE III

Effect of extracts of the ovaries of various vertebrates on the cleavage of Chaetopterus eggs

Animal	Extract no.	Grams ovaries	Ml. sea water	Viscosity 30–40 min. after insem.	% cleavage	Control % cleavage
Frog (*Rana pipiens*)	18	10	20	3	0	—
Frog (*Rana pipiens*)	18	10	40	—	0	98
Frog (*Rana pipiens*)	28	10	30	2	0	100
Frog (*Rana pipiens*)	28	10	60	—	0	100
Frog (*Rana pipiens*)	28	10	120	—	41	100
Salamander (*Amblystoma punctatum*)	38	0.5	5	6	0	99
Salamander (*Amblystoma punctatum*)	38	0.5	10	—	73	99
Chicken	39	26	52	5	2	98
Rabbit	23	2	10	8+	92*	97
Dog	34	2	10	8	3	94
Pig		25	25	<8	0	99
Lamb		14	15	<8	0	99
Cow		90	90	<8	0	100

* Cleavage was delayed 8 minutes.

in relatively pure form, in the hope that they may be able to cause regression of tumors in mice. Preliminary experiments indicate that our crude extracts are capable of causing such a regression, but the results we have obtained thus far vary greatly, and much more work is needed (Heilbrunn, Lippman and Rutman, 1955).

It must not be thought that the ovary is the only organ that contains antimitotic substances. We have made extracts from liver, testis and muscle, and all of these extracts prevent cell division. This is indeed what we would expect, if as we have said, protoplasm usually, perhaps universally, contains substances which have an inhibiting effect on protoplasmic clotting.

SUMMARY

1. Extracts from the ovaries of 14 different species of fishes all have an antimitotic effect on the eggs of the worm Chaetopterus.

2. Extracts from the ovaries of the frog, salamander, chicken, rabbit, dog, lamb, pig, and cow act in similar fashion.

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THE PHYTOPLANKTON OF GREAT POND, MASSACHUSETTS[1]

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Although the plankton flora of moderately deep estuaries has been studied to some degree (Braarud and Bursa, 1939; Cowles, 1930; Gran and Braarud, 1935; Lohmann, 1908; Morse, 1947) very little quantitative study of phytoplankton in very shallow estuaries has been done. Several investigators in Belgium and Holland (Conrad, 1926a, 1926b; Massart, 1920; Van Goor, 1925a, 1925b, 1925c) have studied taxonomically the phytoplankton of very shoal estuaries. The forms they describe are almost entirely flagellates. It would be of great interest to learn whether these taxonomic studies indicate a distinctly different flora composed primarily of flagellates or whether diatoms, as well as flagellates, thrive in very shallow estuaries. Further, in the event that flagellates should dominate in shoal estuaries, it would be of interest to characterize the conditions associated with their dominance.

The Embayment Investigated

Figure 1 shows the form and depth of Great Pond. A narrow gut admits water from Vineyard Sound at one end and a small river, the Coonamessett, empties at the other end. It is divided into a wider seaward portion, the main pond, and a narrower landward portion, the arm. Its depth is nowhere greater than 9 feet at highest high tide. The hydrography of the Pond has been well studied by Barlow (in press), but a few of the salient features are reviewed here. The mean salinity indicates that the Pond water is 83 parts sea water and 17 parts river water. Surface salinity decreases from 32‰ in the Sound to 1–2‰ near the mouth of the River. Bottom salinity decreases from 32‰ to 24‰ near the mouth of the River, indicating marked stratification in the arm. The effect of wind blowing toward the Sound is to move the surface water seaward, resulting in upwelling of deep water and increase of surface salinities to 24‰ or above all the way to the River.

Water samples were taken at the stations indicated in Figure 1 from the shore in February and March and from a rowboat during the rest of the year. Samples were taken within several inches of the surface and approximately ten inches from the bottom. Phytoplankton cell counts and phosphate, turbidity, and salinity determinations were made on each sample.

Methods

Cell counts were made from four to forty-eight hours after samples were collected. Cell concentrations were determined by counting living cells in a very shallow chamber, so that a high power microscope objective could be used. Usually the water sample was concentrated on a smooth-surfaced porcelain filter with a pore

[1] Contribution No. 764 from the Woods Hole Oceanographic Institution.

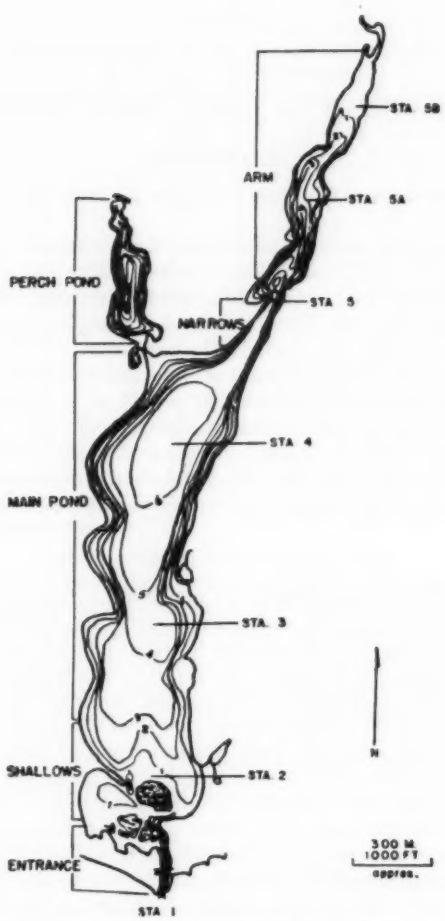

FIGURE 1. The morphometry of Great Pond. Depths are in feet.

diameter of 0.5 μ, manufactured by the Selas Corporation. Occasionally counts were made without filtration. Comparison of filtered and unfiltered counts showed that a large proportion of the cells was lost in filtration. Therefore, all filtered counts were multiplied by 1.81 to give the true concentration of cells in the water. All cells from 2–3 μ in diameter to about 50 μ in diameter were counted.

RESULTS

Figure 2 shows the distribution of total phytoplankton. In the surface waters greater concentrations occurred within the Pond, and this difference was greater

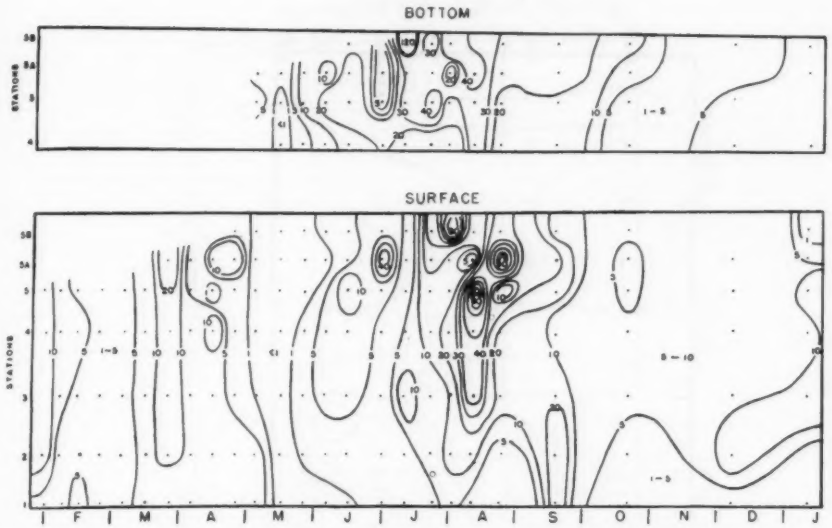

FIGURE 2. The distribution of total phytoplankton. Values $\times 10^6$ are numbers of cells per liter.

during the summer than during the rest of the year. In summer there was, at a maximum, a 20-fold increase from the Sound to the surface waters of the arm. Between mid-September and the end of May, by contrast, the greatest increase from Sound to Pond was four-fold. The highest concentration in the surface waters, over 90,000,000 cells per liter, occurred in the summer at Station 5, well up the Pond. It is noteworthy that much greater fluctuations in cell concentrations occurred during summer than during the rest of the year. This feature was particu-

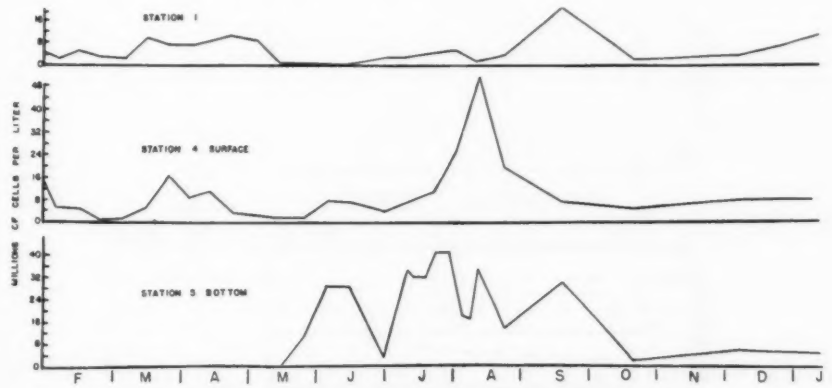

FIGURE 3. The seasonal cycle of total phytoplankton.

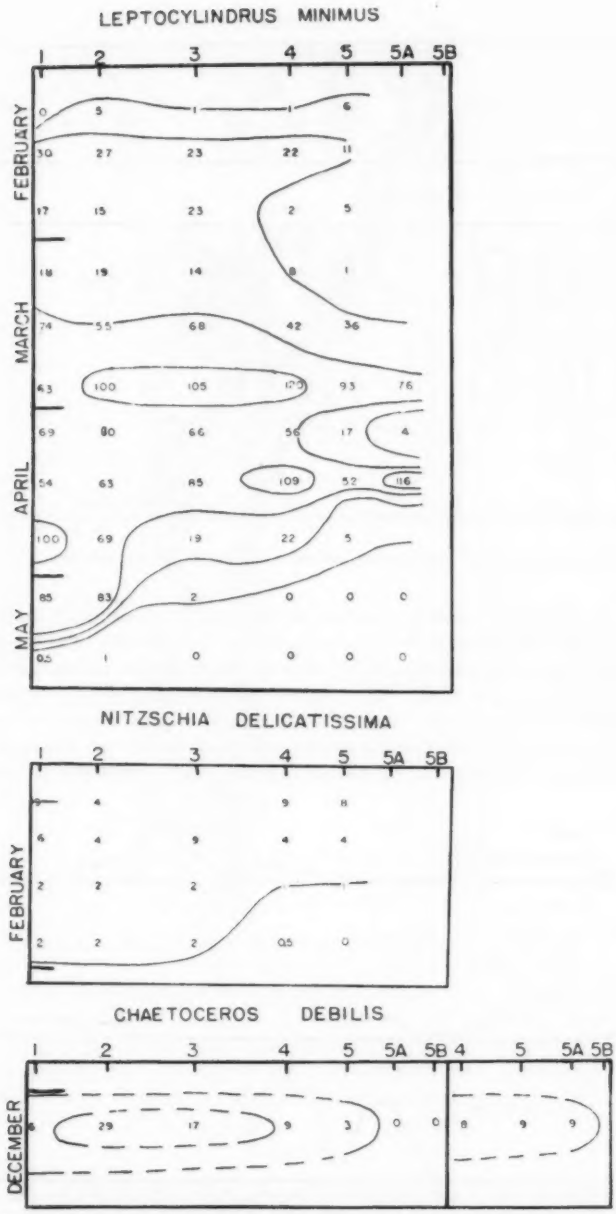

FIGURE 4. The distribution of Sound-derived species. Values $\times 10^3$ are numbers of cells per liter. Station numbers, 1–5b, cross the figure.

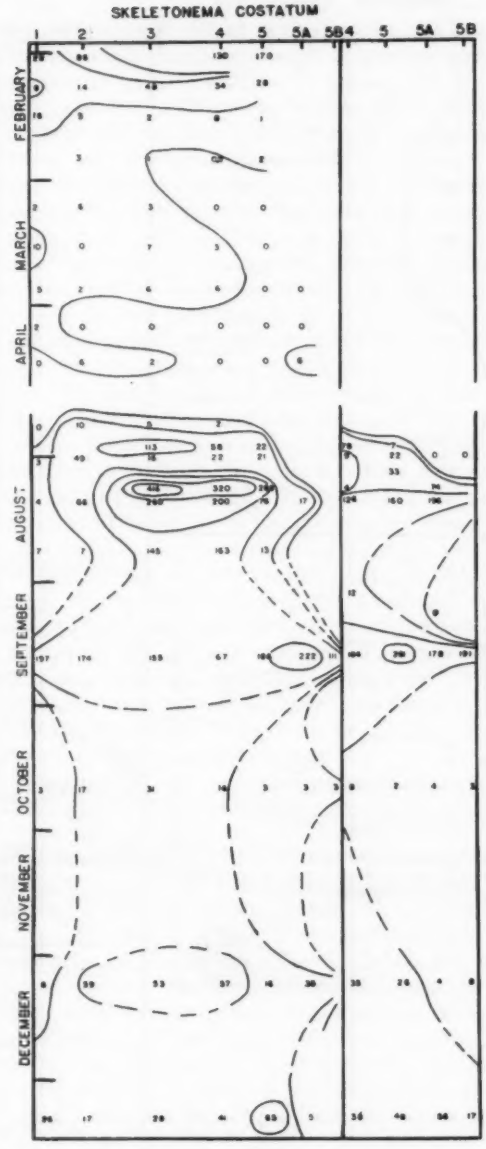

FIGURE 5. The distribution of *Skeletonema costatum*. Values $\times 10^5$ are numbers of cells per liter. Station numbers, 1–5b, cross the figure.

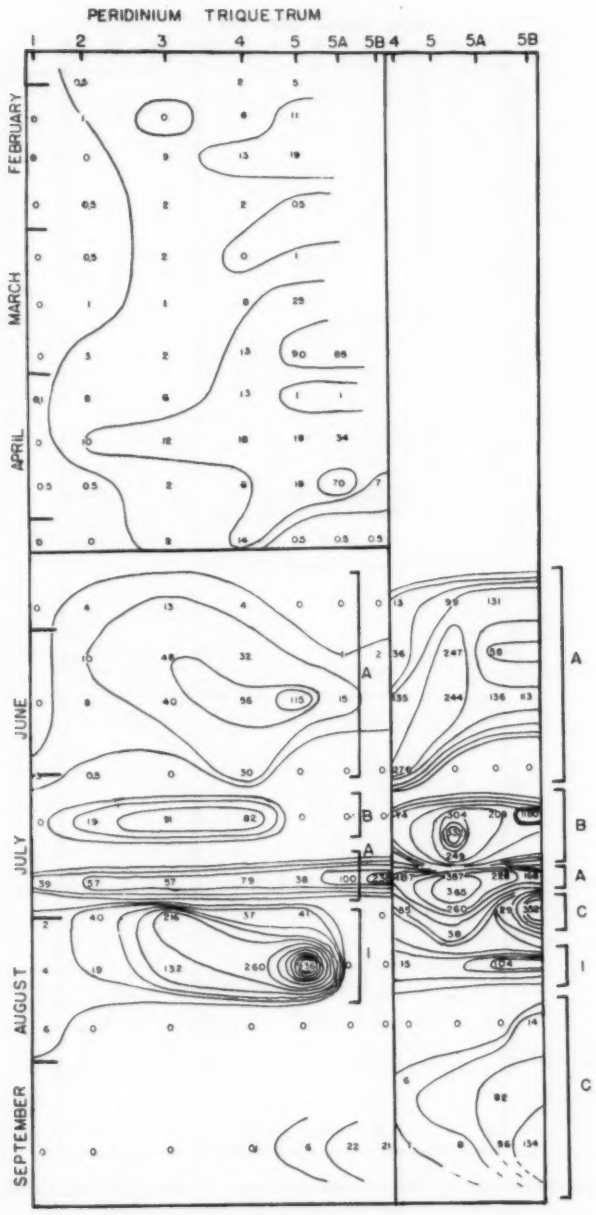
PERIDINIUM TRIQUETRUM
1 2 3 4 5 5A 5B 4 5 5A 5B
FEBRUARY
MARCH
APRIL
JUNE
JULY
AUGUST
SEPTEMBER
A
B
A
I
A
B
A
C
I
C

FIGURE 6.

larly noticeable in the surface waters of the arm, where isolated pools of high concentrations occurred on June 30 and on August 1, 11, and 23.

Cell concentrations along the bottom were, in a gross way, similar to those at the surface, being greater in summer than in fall or winter. However, with the exception of a brief period at the beginning of July, cell concentrations remained at high levels throughout the summer. The highest concentration observed, 120,000,000 cells per liter, was at Station 5b, far from the Sound.

Seasonal cycles for several stations are shown in Figure 3. In the Sound there was a bimodal cycle, with moderate concentrations in late winter and spring and high concentrations in early fall. The surface waters of mid-pond showed a bimodal curve, also, with roughly the same concentrations in late winter and spring, as in the Sound, but, unlike the Sound, with very high concentrations in later summer. Deep water at mid-pond had high concentrations throughout summer and September and moderate concentrations in fall and winter. Sound and Pond both showed lowest concentrations at the end of May.

Figure 4 shows the distributions of three species of diatoms, *Leptocylindrus minimus*, *Nitzschia delicatissima*, and *Chaetoceros debilis*. These were observed only during the cooler months and were found to extend from the Sound far into the Pond. *Leptocylindrus minimus* showed a relatively uniform distribution in the Sound and seaward portion of the Pond. Though concentrations were occasionally somewhat greater in the Pond than in the Sound, they never showed a variation that was more than two-fold. In the surface waters of the arm concentrations were about the same as those in the seaward portion of the Pond on days of upwelling, March 26 and April 15, but were much lower on a normal day, April 5, when no upwelling occurred. In the Sound and main pond *Leptocylindrus minimus* increased rapidly to become the dominant species by February 15, attained a maximum on March 25, and decreased abruptly early in May. *Nitzschia delicatissima* showed a similar distribution to *Leptocylindrus minimus* during February. Its concentrations were about one-tenth those of *Leptocylindrus minimus*. *Chaetoceros debilis* resembled *Leptocylindrus* in distribution.

Skeletonema costatum, the distribution of which is shown in Figure 5, was the only species of diatom that was present during summer as well as during the rest of the year. At times it exhibited a uniform distribution from the Sound far into the Pond, as on September 18, January 10 and February 25. At various other times it was more abundant in the Pond than in the Sound, the variation being eight-fold on October 20, December 7 and January 30 and as high as one hundred-fold in August. During fall and winter it was the dominant species, attaining a maximum on September 18. In February it declined as *Leptocylindrus minimus* became the dominant species. Although *Skeletonema* was not clearly a dominant organism in August, it reached its greatest concentration at that time.

In spring the dinophycean *Peridinium triquetrum* rose to considerable prominence (see Figure 6), its concentrations being as high as one-fifth of those of *Leptocylindrus* on April 15. It was more abundant within the Pond than in the Sound. There was a hundred-fold increase from the Sound to the mid-reaches of

FIGURE 6. The distribution of Pond-derived species. Values $\times 10^6$ are numbers of cells per liter. Station numbers, 1–5b, cross the figure. A represents undetermined chrysophycean, B *Bipedinomonas pyriformis*, C *Nephrochloris salina*, and I *Chaetoceros simplex*.

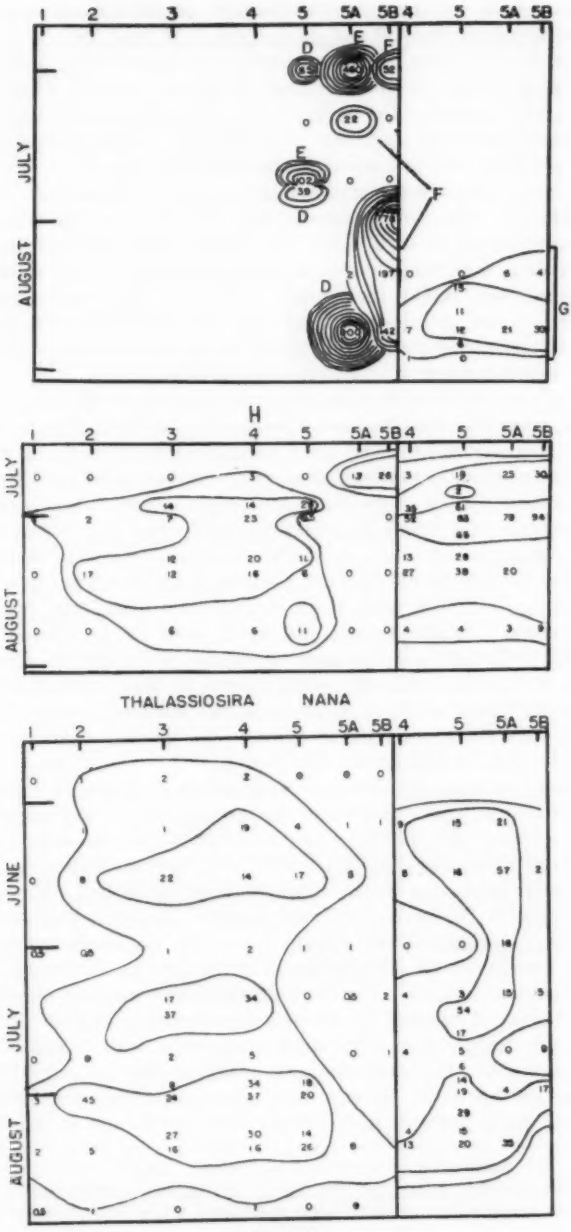
1 2 3 4 5 5A 5B 4 5 5A 5B
JULY
AUGUST
D
E
F
G
H
THALASSIOSIRA NANA
JUNE
JULY
AUGUST

FIGURE 7.

the Pond on March 26. This difference was greater than any observed for the four diatoms so far described in the period between mid-September and the end of May.

Figure 6 shows the distributions of several species found primarily in summer. They were always far more abundant in the Pond than in the Sound. During June the dominant species was an undetermined chrysophycean flagellate. Its numbers were usually greater along the bottom than at the surface. The surface waters at the ends of the Pond had smaller concentrations than the waters of mid-pond, the difference being as much as one hundred-fold. Only in mid-pond did concentrations approach those along the bottom. Abrupt decrease of the chrysophycean was followed in early July by a growth of the flagellate *Bipedinomonas pyriformis*. Its distribution was identical to that of the chrysophycean. By late July *Bipedinomonas* had disappeared completely and the undetermined chrysophycean flagellate was dominant again throughout the Pond. On July 22 the chrysophycean showed an exceptional abundance at the surface of the arm due to upwelling. In August the small diatom *Chaetoceros simplex* became extremely abundant, more so in the surface waters of mid-pond than in the deeper water. On August 11 it showed a two hundred-fold difference between Sound and Station 5. The flagellate *Nephrochloris salina* was moderately abundant in August and September in bottom water at the extreme landward end of the Pond.

Figure 7 shows the distributions of several less important species of summer. The flagellate *Gyrodinium estuariale* and the minute diatom *Thalassiosira nana* exhibited the same type of distribution as described for the undetermined chrysophycean. In the surface waters of the arm contours are close-packed around the flagellate *Chlamydomonas* in the surface waters at the outlet of the river and around the flagellates, *Massartia rotundata* and *Rhodomonas minuta*, farther down the arm. The growth of these species was sporadic and attained very great concentrations. It is noteworthy that when these species grew in the surface waters, no traces of the species were found in the bottom water only five or six feet below.

Discussion

The species can be divided into Sound-, Pond-, and River-derived types. The Sound-derived type was abundant in the Sound and Pond alike. The Pond-derived type was abundant only in the Pond, particularly in the inner reaches and along the bottom. The river type was found primarily in the surface water at the outlet of the River. The two principal Sound-type species were the diatoms *Leptocylindrus minimus* and *Skeletonema costatum*. *Nitzschia delicatissima* and *Chaetoceros debilis* represented well-defined Sound types, but were of minor importance. Principal Pond types were the undetermined chrysophycean flagellate; the heterokontan flagellate *Nephrochloris salina;* the chlorophycean flagellate *Bipedinomonas pyriformis;* the dinophycean flagellates *Peridinium triquetrum, Massartia rotundata,* and *Gyrodinium estuariale;* the cryptophycean flagellate *Rhodomonas minuta;* and the diatoms *Chaetoceros simplex* and *Thalassiosira nana.* The River type was repre-

Figure 7. The distribution of Pond- and River-derived species. Values $\times 10^3$ are numbers of cells per liter. Station numbers, 1–5b, cross the figure. D represents *Massartia rotunda,* E *Rhodomonas minuta,* F *Chlamydomonas* sp., G *Gyrodinium dominans,* and H *Gyrodinium estuariale.*

sented by chlorophycean flagellates, consisting of two unidentified species of *Chlamydomonas*.

The Sound-derived species thrived during fall, winter, and spring primarily and in summer to a small degree. The Pond-derived species flourished in summer primarily and in spring to a small degree. All of the Sound-derived forms were nonmotile; the majority of the Pond-derived forms were motile.

During the fall, winter, and spring the water of the Sound, which fills 83% of the Pond at any time, carried its plankton far into the Pond. Within the estuary changes in this plankton were slight or negligible, indicated by the small difference in total plankton between Sound and Pond, by the change from dominance of *Skeletonema* to *Leptocylindrus* simultaneous in Sound and Pond, and finally by the intrusion of Sound species far into the Pond. During summer the Sound water carried with it little plankton, and once within the estuary the identity of this plankton was lost to a large degree. Plankton from the River could be identified only in the surface waters where the River emptied into the Pond. Throughout the greater portion of the Pond an indigenous plankton flourished.

TABLE I

Concentrations of diatoms and flagellates and statistics of physical features along the length of Great Pond

	Diatoms $\times 10^4/1$	Flagellates $\times 10^4/1$	Six-hour mean tidal velocity, knots	Salinity difference between surface and bottom, ‰
Sound	34	43	1.60	—
Mid-Pond, Station 4	633	598	0.01	6.38
Arm, Station 5a	319	1492	0.02	17.90

During the transport of water through the Pond, rapid changes in the plankton occurred in summer, as indicated by the abrupt changes in dominant species from place to place on the same survey. In the surface waters of the arm such changes caused large fluctuations in total phytoplankton. These changes indicate a rapid growth rate. During the remainder of the year abrupt changes in dominant species and extreme variations in total phytoplankton did not occur and hence growth rates were less than in summer. Thus, when conditions for growth in the Pond were good, Pond species rather than Sound species flourished. When growth conditions were less favorable, during the cool months, the plankton of the Sound drifted into the Pond, replacing the endemic flora that had previously flourished.

It has been pointed out that during summer the dominant members of the plankton included seven flagellates and three diatoms. In order to account for the preponderance of motile forms within the Pond, its association with low current velocities and great vertical stability of the water will be investigated. Averages of the dominant species as flagellates or diatoms are given for various stations in Table I. Surface and bottom values are averaged for stations 4 and 5a. Current velocities were taken from surface current measurements for Vineyard Sound one mile south of Great Pond (U. S. Coast and Geodetic Survey, 1949) and were computed for Great Pond by dividing the intertidal volume landward of a station by the cross-sectional area at the station. Surface current measurements may be

higher than computed currents estimates by as much as 50% (Riley, 1952). The salinity difference between surface and bottom was the average difference of all observations during summer. Both diatoms and flagellates increased from the Sound to mid-pond, but flagellates increased at the expense of the diatoms from mid-pond to the headwaters. Tidal currents were much less from mid-pond to headwaters than in the Sound; while the vertical salinity gradient became progressively greater up-pond.

The moderate success of the motile cell from Sound to mid-pond can be attributed to a decrease in vertical turbulence, as a result of lessened tidal current. The greater success from mid-pond to the headwaters can be attributed to further decrease in turbulence, as a result of both weak currents and marked stability. Settling out of non-motile cells in the quiet waters of the Pond, particularly the arm, would place the motile cell at an advantage. In addition, cells destined to settle out would have less time to divide and replace the stock in the shallow waters of the Pond than in the deep waters of the Sound, so that the motile cell would be at a still greater advantage in the Pond. Thus, when growth conditions favor an endemic flora, the quietness and shoalness of the Pond water should favor motility as a characteristic of the flora.

The Pond flora may have been better adapted than the Sound flora to lowered salinity, high temperature, or nutrient products of the intermediate metabolism of the pond water. Adaptations of these sorts may have been effective in favoring the successive blooms of different species and in fostering the occasional dominance of non-motile forms.

In conclusion, the summer conditions at Great Pond furnish an example of the dominance of flagellates rather than diatoms in the phytoplankton. This dominance appears to result in part from a coupling of good growth conditions, which foster an endemic flora, with a quiet, very shallow habitat, which should favor the non-settling, motile cell.

The author wishes to express the greatest gratitude to Dr. A. C. Redfield and to Dr. W. R. Taylor for their help during this study. The best of thanks go to Dr. John Barlow, for use of salinity data and help in collecting samples, and to Mr. Harry Turner, for use of the chart of Great Pond prepared by him.

Summary

1. Determinations of phytoplankton concentrations were made throughout 1950 in Great Pond, a small estuary near Falmouth, Massachusetts.

2. Greater concentrations occurred within Great Pond than in the ocean water at its entrance. This difference was greatest in summer.

3. During fall, winter, and spring the same species populated the waters of the ocean and the Pond, but during the summer an assemblage of species flourished within the Pond that was never found in the ocean water and was not characteristic of the river water.

4. The Pond-derived flora of summer contained a large proportion of flagellates, whereas the Sound-derived flora was composed completely of diatoms.

5. The preponderance of motile forms within the Pond during summer was attributed to good growth conditions, which stimulated an endemic flora, and to shoal, quiet water, which should favor the non-settling type of cell.

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ON THE CHEMICAL COMPOSITION OF THE BROWN ALGA HIMANTHALIA ELONGATA (L.) S. F. GRAY

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In recent years the commercial importance of many seaweeds has resulted in considerable work being carried out on the chemical composition of marine algae (Black, 1953). *Himanthalia elongata,* although a common dioecious fucoid along the coasts of northern England, does not occur in sufficient quantities to render it of commercial importance. As a result it has been little investigated. Colin and Ricard (1930) first recorded the chemical composition of *Himanthalia,* but their work is of little value since no indication of the size or condition of the plants analyzed was given. Moss (1952), however, noted that during the spring a variation in chemical composition occurred with the stage of development of *Himanthalia* collected at the same time from the same habitat. No distinction of sex was made in the samples, although for *Fucus vesiculosus* Moss (1950) found that the male receptacles were higher in total nitrogen than the female receptacles.

The present work is concerned with the chemical constituents of both male and female plants of *Himanthalia* and with the variation of such constituents along the length of the plant.

Materials and Methods

Samples of *H. elongata* were collected from St. Mary's Island, Northumberland at low water as the tide receded. All samples were returned to the laboratory in sea water thus ensuring that the plants, prior to analysis, were fully turgid. For analysis samples were sorted into developmental and sexual stages. Each sample contained at least 100 individuals. Fresh weight determinations were made after removing the adhering moisture from the surface of the plants with cheese cloth. The plant material was loosely packed in shallow aluminum trays, placed in an electric oven through which a current of air was forced. The material was dried for one hour at 90° C. (to inactivate enzymes) and then for a further 23 hours at a temperature range of between 60 and 65° C. On cooling to room temperature the dried material was ground in a Christie & Norris No. 8 Laboratory Mill, fitted with a $\frac{1}{64}$-inch perforated screen, giving a powder which practically all passed 90 mesh. Dry weight determinations were made on 0.1–0.2 gm. samples of the above dried milled weed.

Total nitrogen estimations were obtained by the micro-Kjeldahl method of Kelley, Hunter and Sterges (1946) using a Markham Still for distillation. The soluble non-protein nitrogen was extracted from the dried material with water for 6 hours at laboratory temperature with constant shaking, then stored at 5° C. for 18 hours with occasional shaking. After filtering, the proteins were precipitated with sufficient trichloracetic acid to produce a 2.5% solution. The extract was

filtered through Whatman 42 filter paper and made up to 250 ml. This method of extraction gave consistent results for total non-protein nitrogen. The soluble non-protein nitrogen was determined using the method of Langer (1952). Of the soluble non-protein constituents, volatile base and amide nitrogen values were obtained by the method of Channing and Young (1953). The free α-amino nitrogen was estimated using the technique employed by Sobel, Hirschman and Besman (1945). Peptide nitrogen was determined using the method of Haas, Hill and Russell-Wells (1938); Sideris and Young (1946).

The carbohydrate and related constituents were determined by the methods of Cameron, Ross and Percival (1948).

Inorganic ash content of the dried material was estimated after preliminary sulphuric acid treatment as described by Piper (1950).

Free amino acids were extracted from the dried milled weed (one gm.) by shaking three times with 75% ethanol (100 ml.). The solution was centrifuged after each extraction and the combined extracts passed through Zeo-Karb 225 and the free amino acids and peptides eluted with ammonium hydroxide (1 N). The eluates were dried *in vacuo* over calcium chloride and taken up in HCl (0.1 N) and chromatographed. Combined amino acids in the dried material (0.5 gm.) were hydrolyzed in a sealed tube with HCl (6 N; 5 ml.) for 48 hours at 105° C. Excess acid was removed by drying *in vacuo* over calcium chloride and sodium hydroxide. Samples were ion exchanged as above and similarly chromatographed. For the detection of tryptophane, alkaline hydrolysis with barium hydroxide (saturated at room temperature) was used.

Free sugars were extracted from the dried material (3 gm.) after refluxing with 75% ethanol (100 ml.) for 6 hours. After filtration the extract was passed through the ion exchange resins Zeo-Karb 225 and De-Acidite E, reduced in volume and aliquots used for paper chromatography. Sugars on hydrolysis were determined by hydrolyzing the dried milled weed (one gm.) with HCl (50 ml.; 2 N) for 8 hours using a reflux condenser. Following filtration the extract was subjected to ion exchange resins as for free sugars and chromatographed.

Extraction of the organic acids was accomplished by shaking the dried milled weed (3 gm.) with water (100 ml.) for 6 hours. The mixture was filtered and 80% ethanol added to precipitate protein. The precipitate was removed by centrifugation and the supernatant passed through Zeo-Karb 225 followed by De-Acidite E. The acidic compounds were eluted from the latter resin with ammonium hydroxide (1 N).

Methods in paper chromatography were those described by Block (1952) and Ranson (1955).

Results

1. *Qualitative determination of some chemical constituents*

a) Amino acids, free and combined

The presence of only a few free amino acids, namely alanine, aspartic and glutamic acids, was detected on one and two dimensional chromatograms. The normal plant amides asparagine and glutamine were not detected. On hydrolysis with HCl (6 N) for 48 hours the alcoholic extracts gave compact spots corresponding

to aspartic, glutamic, alanine and traces of glycine, serine and the leucines, suggesting the presence of these acids in peptide formation. The intensity of the glutamic and alanine spots was greatly increased after hydrolysis, suggesting that they occur in peptides as well as in the free state. Other alcoholic extractions of different developmental stages of *Himanthalia* gave similar results.

A chromatographic analysis of the peptide fraction precipitated from the water-soluble non-protein extracts with mercuric acetate and treatment with H_2S, as employed by Haas, Hill and Russell-Wells (1938), after hydrolysis revealed the presence of the following amino acids: glutamic (strong), alanine (strong), aspartic (weak-strong), serine (weak), glycine (weak), leucine (trace), isoleucine (trace).

In acid and alkaline hydrolysates of the dried milled weed from all stages of development the following amino acids were detected: alanine, arginine, aspartic, cysteic, glycine, glutamic, histidine, iso-leucine, leucine, lysine, methionine, methionine sulphoxide, phenylalanine, proline, serine, threonine, tryptophane, tyrosine and valine. In the majority of samples analyzed histidine was present only in traces, its presence being confirmed with the Pauly diazotinized sulphanilic reagent (Consden *et al.*, 1946). The isatin dip method of Jepson and Smith (1953) confirmed the presence of proline and the absence of hydroxyproline. The copper carbonate technique for the detection of non α-amino acids (Crumpler and Dent, 1949) failed to show the presence of either β-alanine or γ-amino butyric acid in the free state or in combination.

b) Carbohydrates

Very little sugar could be detected in the free state. Spots with R_F values similar to fucose and galactose were obtained. Glucose, fucose, arabinose, xylose, and galactose were found to be present on chromatograms of the acid-hydrolyzed weed samples. The sugar alcohol mannitol was detected on both sugar chromatograms.

c) Organic acids

Very few organic acids were detected in extracts of *Himanthalia*. Citric acid was the chief organic acid detected. In some cases traces of malic acid were found to be present. No other acids of the Krebs cycle were detected. On all chromatograms a large acidic spot was found to exist in appreciable quantity. In tertiary amyl-formic acid-water solvent, the $R_F = 0.20$, in the propanol-NH_3 solvent, the $R_F = 0.51$. With silver nitrate reagent it gave an intense white color on a brown background. It was negative to ninhydrin and Hane's phosphate reagent. On the chromatograms it ran in close association with standard gluconic acid. The large quantities of alginic acid present in the weed samples leads one to think that it may be mannuronic acid, which would be the precursor of the larger alginic acid molecule. Unfortunately a standard sample of mannuronic acid could not be obtained. Another acidic spot was found to be orthophosphate.

2. *Variation in nitrogenous constituents*

During July, 1953, samples of *Himanthalia* were collected from St. Mary's Island. After preliminary sorting, drying and grinding these plants were analyzed

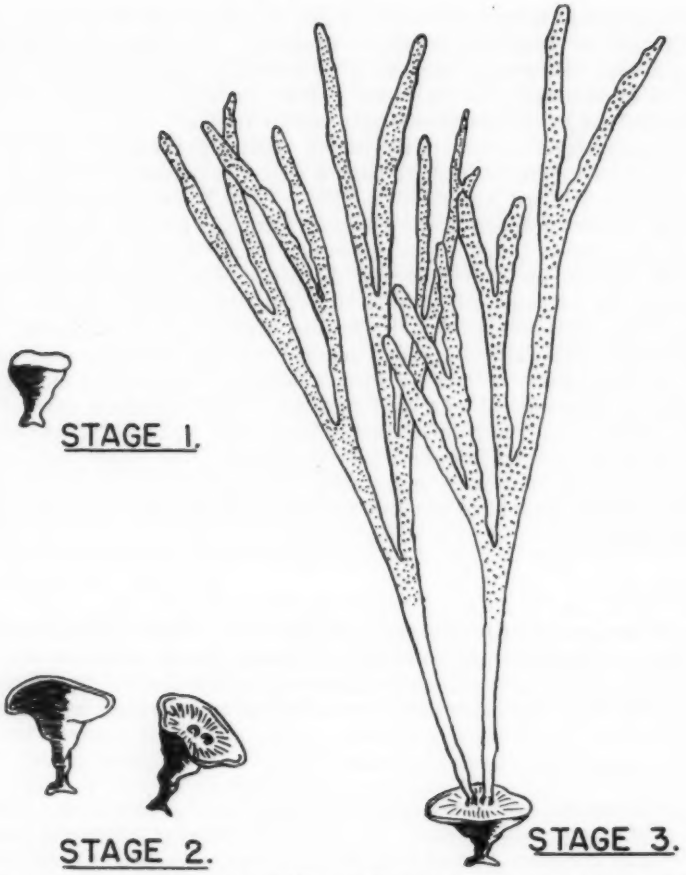


FIGURE 1. Stages of development of Himanthalia used in the chemical analysis, July, 1953: Stage 1. Young vegetative buttons, 1–1.5 cm. in length and tubular in form. Stage 2. Fully grown vegetative buttons. Stage 3. Mature plants composed of buttons, from which were given off long mature receptacles some 50–60 cm. in length. The receptacles were separated from the buttons, and further sorted into male and female for separate analysis.

for total nitrogen, protein nitrogen and non-protein nitrogen. The stages of development used in this analysis are shown in Figure 1.

The results (Table I) are interesting insofar as they show that the male receptacles contain higher nitrogen values than the female receptacles. The higher nitrogen content of the male is associated with the protein fraction. The vegetative plants, *i.e.*, the buttons, contain a higher percentage of soluble non-protein nitrogen than the mature buttons in stage 3. This appears to consist mainly of peptide and free amino acid nitrogen.

TABLE I

Variation of nitrogen in plants from St. Mary's Island, July, 1953

i) Total nitrogen, protein and non-protein nitrogen

Stage of development	Total N	Protein N	Non-protein N	Non-protein N as % total N
1. Buttons	6.75	5.84	0.91	13.5
2. Buttons	12.30	9.85	2.45	19.9
3. Buttons	12.15	10.92	1.23	10.1
Female receptacles	13.45	10.54	2.91	21.6
Male receptacles	15.00	12.12	2.89	19.3

ii) Soluble non-protein constituents

Stage of development	Volatile base N	Peptide N	α-Amino N
1. Buttons	0.20	0.46	0.43
2. Buttons	0.62	1.39	0.59
3. Buttons	0.31	0.97	0.32
Female receptacles	0.63	1.54	0.93
Male receptacles	0.60	1.40	0.69

3. *Further analysis of male and female receptacles*

During September, 1954, when plants were ripe, male and female individuals were collected and the receptacles analyzed separately. The results are given in Table II.

In order to obtain antherozoids and oogonia for analysis, male and female receptacles were separately washed with filtered sea water and allowed to dry out for several hours. During this process gelatinous masses of gametes appeared at the ostioles of the conceptacles. The plants were then placed in separate Pyrex glass containers with filtered sea water and the plants allowed to extrude their gametes. The gametes were separated from the sea water by centrifugation, dried at 90° C.

TABLE II

Analysis of male and female receptacles, Sept., 1954

	Male receptacles	Female receptacles
Mean height	84.7 cm. per unit plt.	86.5 cm. per unit plt.
Mean width	0.8 cm. per unit plt.	0.8 cm. per unit plt.
Fresh weight	122.3 gm. per unit plt.	119.0 gm. per unit plt.
Dry weight	15.6 gm. per unit plt.	15.2 gm. per unit plt.
Total nitrogen	19.7	16.6
Protein nitrogen	14.8	12.0
Non-protein N	4.9 mg. per gm. dry weight	4.6 mg. per gm. dry weight
Mannitol	65.1	66.8
Alginic acid	132.4	178.9

TABLE III

Analysis of male and female gametes, Sept., 1954

Constituent	Male gametes (mg./gm. dry wt.)	Female gametes (mg./gm. dry wt.)
Total nitrogen	69.33	25.97
Protein nitrogen	55.81	13.83
Non-protein nitrogen	15.52	12.14
Total ash	826.60	819.60

for one hour, followed by 23 hours at 60° C. An analysis of the nitrogenous constituents and total inorganic ash is given in Table III.

The results of these two analyses show that between the male and female receptacles there is little difference in size and weight. The protein nitrogen is higher in the male than in the female, thus confirming the results found for the receptacles collected in July, 1953. The male gametes are appreciably higher in protein content than the female gametes, which suggests that the high protein content of the male receptacles is in all probability due to the high protein content of the gametes. The exceptionally high ash content of the gametes is of great interest. The alginic acid is higher in the female receptacles than in the male.

4. *Variation of chemical constituents along the length of the plant*

One hundred plants were collected from St. Mary's Island during April, 1954, and divided into three regions for analysis of the various chemical constituents. The divisions were as follows:

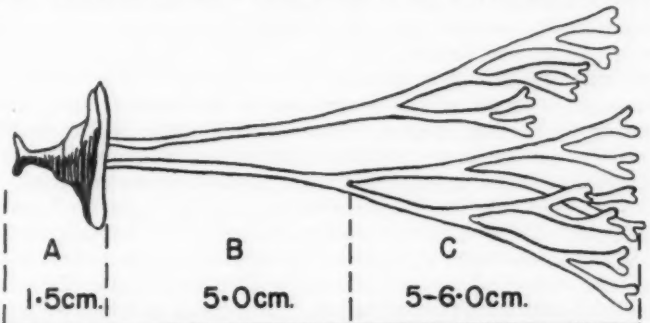
Region A. Mature buttons of the plant.
Region B. Sterile base of receptacle, the length of which was approximately 5 cm. from the surface of the button.
Region C. Remainder of the receptacle. This region contained developing conceptacles which are differentiated behind the growing apices. Length of region varied from 5.0 to 6.0 cm.

The results in Figure 2 indicate that variations occur along the length of the plant. Of the nitrogenous constituents analyzed, the young developing receptacle tips and conceptacles of region C possess higher values than the base of the receptacle (region B) which is sterile. The vegetative button (region A) possesses considerably lower amounts of nitrogen than those recorded for either region of the receptacle. The tips and base of the receptacle are higher in mannitol than the vegetative button. The button, however, is higher in alginic acid and laminarin content. The sterile base of the receptacle contains more alginic acid than the tips of the receptacles.

GENERAL DISCUSSION

The qualitative determination of some of the chemical constituents recorded for *Himanthalia* is in accordance with the findings of other workers. The detection of only a few amino acids in the free state is to be found in other marine algae (Channing and Young, 1953; Coulson, 1953a; Smith and Gordon Young, 1953). The

presence of peptides, particularly of glutamic acid and alanine, affords further evidence that marine algae differ from fresh water algae and land plants in that they show a preference for peptides in place of amino acids (Dent, Stepka and Steward, 1947; Steward and Thompson, 1950; Fowden, 1951). The typical land plant



CONSTITUENT	BUTTON	BASE of RECEPTACLE	UPPER RECEPTACLE
TOTAL N	13·51	20·25	28·70
PROTEIN N	11·87	15·56	20·29
NON PROTEIN N	1·64	4·79	8·41
VOL. BASE N	0·39	0·66	0·77
FREE AMINO N	0·50	1·20	1·19
PEPTIDE N	0·71	1·59	2·12
ALGINIC ACID	215·40	166·80	119·00
MANNITOL	60·77	74·38	79·62
LAMINARIN	21·18	8·15	9·61

(mg. per gm. dry wt.)

FIGURE 2. Variation of constituents along length of plant, April, 1954.

amides asparagine and glutamine were not detected in *Himanthalia*. The amino acids present in the hydrolysates of the marine alga were similar to those found by other workers for the Phaeophyceae (Channing and Young, 1953; Coulson, 1953b; Smith and Gordon Young, 1953), and for fresh water algae (Fowden, 1954). They are similar to those in land plants. The sugars fucose and galactose, and the sugar-alcohol mannitol were detected in the free state. The recent work of Lindberg (1953) and Lindberg and Paju (1954) has shown the presence of mannitol and mannitol glucosides to occur in other fucoids. It is realized, however, that the presence of fucose and galactose may be the result of polysaccharide hydrolysis during the drying process. The sugars glucose, fucose, arabinose, xylose and galactose present in the acid hydrolysates are in keeping with the results of other workers (Connell, Hirst and Percival, 1950; Percival and Ross, 1950; Ross, 1953; Dewar, 1954), in that they are the component sugars of laminarin, fucoidin and cellulose. Little work has been published on the organic acids of marine algae apart from the work of Créach (1952), who detected citric acid in a number of fucoids. The presence of citric acid and traces of malic acid found in *Himanthalia* are commonly found in land plants. The presence of free orthophosphate has been noted for other algae (Blinks, 1951).

The male receptacles of *F. vesiculosus* were shown by Moss (1950) to be higher in nitrogen content than the female receptacles. For *Himanthalia* similar results have been obtained, the male receptacles containing more nitrogen, particularly protein nitrogen, than did the female receptacles. The male gametes of *Himanthalia* were also found to contain more nitrogen than female gametes. This is similar to results published by Sosa-Bourdouil (1940), who, for *F. vesiculosus*, found the male gametes to contain twice as much nitrogen as the female gametes on a dry weight basis. The high concentration of nitrogen in the male gametes seems therefore to be the factor contributing to the high nitrogen content of the male receptacles.

The analysis of the chemical constituents along the length of the plant showed the tissues of mature buttons to be lower in nitrogen and mannitol but higher in alginic acid and laminarin than were the receptacles on the same plant. The apical regions of the receptacles were high in mannitol and nitrogen content, particularly protein. The basal sterile region of the receptacle was similarly high in mannitol, but lower in nitrogen than the uppermost region. The higher soluble non-protein nitrogen content of the upper receptacle region is, no doubt, correlated with the synthesis of protein for growth and gamete formation. The results may be compared with those of Moss (1950), who for *F. vesiculosus* found that the young receptacles were higher in nitrogen content than the vegetative regions of the thallus, which is in contrast with *Himanthalia* for in this species the fertile tissue was higher in nitrogen content than the vegetative button. Jacobi (1954) has recorded high nitrogen values for the apical regions of *F. vesiculosus* throughout the year. For *Laminaria saccharina*, the same author found a higher nitrogen content in the meristematic region of the thallus than in the more mature tissues of the frond. The findings of Moss (1950) and Jacobi (1954), together with the present author's results for *Himanthalia*, indicate that actively growing regions and regions of reproductive development are higher in nitrogen content than the more mature vegetative tissues of the plant.

The work in this paper forms part of the programme of research and development on marine algae by the Institute of Seaweed Research and the author is indebted to the Institute for permission to publish. The author also wishes to thank Dr. Betty L. Moss, under whose supervision this work was carried out.

Summary

1. The qualitative determination of amino acids, sugars and organic acids in *Himanthalia elongata* has been carried out.

2. Mature vegetative tissues were found to be lower in nitrogen content than actively growing reproductive tissues.

3. The high nitrogen content of the male receptacles was correlated with the higher protein nitrogen content of the male gametes.

4. Vertical gradients of organic constituents were found to occur along the length of the individual plant. The mature button was lower in nitrogen and mannitol but higher in alginic acid and laminarin than the receptacles of the same plant. The apical regions of the receptacles were high in mannitol and nitrogen content, particularly protein. The basal sterile region of the receptacle was similarly high in mannitol, but was lower in nitrogen than the uppermost region.

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THE EFFECT OF SEX AND ALLATECTOMY ON THE OXYGEN CONSUMPTION OF THE THORACIC MUSCULATURE OF THE INSECT, LEUCOPHAEA MADERAE[1]

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Previous results of metabolic studies in a variety of insect species clearly illustrate the difficulty in making generalizations (Sacktor, Thomas, Moser and Block, 1953). Thus it is not too surprising that, in contrast to the work of Barron and Tahmisian (1948) in *Periplaneta americana*, no sex difference was found in the present study regarding the endogenous oxygen consumption of the musculature of *Leucophaea maderae*. A further, more interesting result is that the thoracic muscles of both sexes of *Leucophaea maderae* increase their oxygen consumption after allatectomy. This finding is in seeming contrast to that of Thomsen (1949) who observed a decrease in the oxygen consumption of the whole intact fly, *Calliphora erythrocephala*, following allatectomy. However, this discrepancy may perhaps be explained on the basis of the work of L'Hélias (1953) who found an increase in the carbohydrate content of tissues and a decrease in blood sugar of *Dixippus morosus* after the removal of the corpora allata (see Discussion).

Preliminary tracer experiments were undertaken in order to ascertain whether there was any correlation, *in vivo*, between the increased oxygen consumption and phosphorylation, but they were terminated before any definite conclusions could be drawn.

Methods

The determination of oxygen consumption (QO_2) was carried out at 26° C. in a conventional Warburg apparatus, each vessel containing a total volume (including muscle and KOH) of 3 ml. of Bělař's solution (.9% NaCl; .02% KCl; .02% $CaCl_2$) buffered at pH 6.8 with .01 *M* phosphate. The thoracic muscles, weighing approximately 150 mg., of a single animal were kept on ice (1–3° C.) in Bělař's solution, without glucose or other substrate, weighed, separated with the aid of needle probes into very small pieces, and placed in the Warburg vessels. Oxygen consumption measurements were initiated, after a 15-minute equilibration period, in less than an hour after the muscles were dissected out. The moisture content, determined on normal animals of both sexes and allatectomized animals at the end of a few of the Warburg runs, averaged 80%. Allatectomy was performed soon after emergence and the operated animals were used two to three months afterwards. The controls were always of the same adult age.

The preliminary tracer experiments were carried out *in vivo*. Two microcuries

[1] The work was supported by grants from the American Cancer Society and The Anna Fuller Fund administered by Dr. Berta Scharrer.

of a high activity solution of neutralized ortho sodium phosphate ^{32} were injected into the body cavity of each of the animals. They were sacrificed 24 hours later, the thoracic muscles and the gut were dissected out, rinsed in several changes of phosphate-free Bělař's solution, blotted, weighed, and frozen on dry ice, prior to homogenizing in iced 10% trichloracetic acid. The phosphorus was fractionated by the method described by Schneider (1945).

Counting was carried out with a thin window (1.4 mg.) G-M tube on aliquots of the ashed fractions, containing fairly uniform amounts of phosphate.

Results

Table I shows that there was no significant difference between the sexes in the QO_2's of the thoracic muscles, 11 males averaging $3.25 \pm .28$ and 10 females averaging $3.35 \pm .28$ cubic mm./mgm. dry wt./hour. Deviation is expressed as standard error. Inasmuch as the oxygen uptake of the muscles was linear for about

Table I

Oxygen consumption of roach muscle Leucophaea maderae
Male versus Female

Experiment No.	Males	QO_2	Females
1	2.7		2.9
2	2.5		2.1
3	3.0		2.5
4	2.8		3.9
5	1.7		4.6
6	4.7		3.3
7	3.9		2.3
8	4.1		4.1
9	4.0		3.1
10	4.2		4.7
11	2.2		
Average	$3.25 \pm .28$		$3.35 \pm .28$

50 minutes, the QO_2 values were calculated on the basis of the average oxygen consumption at 30 minutes. In a few trial experiments, we were able to confirm the results of Barron and Tahmisian (1948) on *Periplaneta americana*, in which they found a larger oxygen consumption in male thoracic muscles than in females.

In another experimental series dealing with allatectomized as compared with normal control males and females, the muscles of 18 normal animals had a QO_2 of $3.50 \pm .22$ while 18 allatectomized males and females averaged $4.26 \pm .20$. These results are shown in Table II. The 20% difference is significant, as a "t" test gives a P of approximately .02.

The two series of experiments (male vs. female and normal vs. allatectomized) were done at widely separate times, and as slight advantage, statistical or otherwise, would be gained by pooling the two series of normal animals, the calculation was not made.

In preliminary, *in vivo*, tracer experiments, utilizing P^{32}, the 24-hour uptake into the acid-soluble (A.S.), phospholipid (P.L.), nucleic acid (N.A.) and residual phosphate (R.P.) fractions was determined. Eight animals, five normal and three

TABLE II

Oxygen consumption of roach muscle Leucophaea maderae
Normal versus Allatectomized

Experiment No.	Normal QO_2	Allatectomized
1	4.3	4.8
2	2.5	3.7
3	3.9	4.5
4	4.6	5.8
5	3.3	4.2
6	2.3	3.8
7	4.1	4.4
8	3.1	4.2
9	4.7	5.9
10	3.0	4.1
11	2.8	3.8
12	1.7	2.8
13	4.7	5.2
14	3.9	4.4
15	4.1	4.0
16	4.0	3.3
17	4.2	4.8
18	2.2	3.0
Average	3.50 ± .22	4.26 ± .20

allatectomized, were used. Tissues were rinsed thoroughly to remove adhering tracer phosphorus. In terms of relative specific activities, that is, specific activity ratios of the insoluble fractions to the acid-soluble fraction, the ratios in the gut were two to five times higher than those of similar fractions of muscle. The higher relative specific activities of gut indicate that phosphate is metabolized at a higher rate in all fractions of gut than in those of muscle. This higher metabolic activity is supported by preliminary experiments in which the oxygen consumption of gut was also shown to be higher than that of muscle. However, as it was difficult to be certain of complete removal of normal gut bacteria, the reliability of the QO_2 values was questionable at that time.

TABLE III

Uptake of radioactive phosphorus into muscle of normal and allatectomized Leucophaea maderae

Date	Animal	Specific activity							
		Acid soluble	A.S./Blood	P.L.	P.L./A.S.	N.A.	N.A./A.S.	P.P.	P.P./A.S.
1–14–54	Normal	255	.66	12.4	.048	41.0	.160	37.4	.146
1–14–54	Normal	246	.64	10.8	.044	44.6	.182	39.0	.158
1–14–54	Allatectomized	377	—	14.0	.037	61.0	.161	82.8	.218
7–9–54	Normal	105	.44	5.0	.047	16.6	.158	14.0	.133
7–9–54	Normal	293	.45	11.6	.040	48.0	.164	51.6	.178
7–9–54	Normal	134	.54	7.2	.053	24.2	.181	24.4	.182
7–9–54	Allatectomized	154	.55	6.9	.045	29.9	.194	28.0	.181
7–9–54	Allatectomized	165	.42	8.1	.049	41.0	.248	33.9	.206

However, in the muscles, as shown in Table III, no significant differences could be found in the AS or PL fractions between normal and allatectomized roaches. For example, the relative specific activities of the phospholipids PL/AS of the normals average .046 ± .004 while those of the allatectomized average .044 ± .005. Error is expressed here as standard deviation.

The specific activities of the N.A. and P.P. fractions appear closely related, which may mean that the two groups of compounds are metabolically related, that they have reached a similar equilibrium value, or that the method of separation is inadequate. However (although no statistical significance may be attached to the fact) the average of the relative specific activities of the allatectomized animals in the N.A. and the P.P. fractions, separately, is higher than that of the controls.

Discussion

The increased oxygen consumption of the muscles of the allatectomized animals (Table II) indicates that the removal of the corpus allatum hormone may result in an elevation of the endogenous substrate concentration (glucose and glycogen) and/or the concentration of some oxidative enzymes in insect muscle. In allatectomized *Dixippus morosus,* L'Hélias (1953) found an elevated carbohydrate content in the tissues and a lowered blood sugar. If comparison of these two forms of insect is possible, one would, by analogy, expect a similar increase in the carbohydrate stores in the muscles of allatectomized *Leucophaea maderae,* which when removed from the ***in vivo*** situation and thrown upon its endogenous resources ***in vitro,*** would result in a higher oxygen consumption than that of the normal control muscles under the same circumstances. Furthermore, if the lowered blood sugar of *Dixippus morosus* holds for *Calliphora* as well, it would explain the results of Thomsen (1949) showing a lowered oxygen consumption of living allatectomized flies, for in the living animal the tissues might be constrained to the use of the lowered blood sugar and would consequently respire at a lower rate than controls with a normal blood sugar.

This hypothesis is, of course, tentative in view of the metabolic variation obtaining even in closely related species of insects.

The absence of a sex-correlated difference in muscle oxygen consumption shown here, although not obtaining in *Periplaneta americana* (Barron and Tahmisian, 1948) could have been predicted from the succinoxidase contents of muscles of males and females of these species, *Leucophaea maderae* and *Periplaneta americana,* as the results of McShan, Kramer and Schlegel (1954) which appeared while this work was in progress showed that there is no sex difference in the content of this oxidative enzyme in *Leucophaea maderae,* as compared to the three-fold difference found by Harvey and Beck (1953) in *Periplaneta americana.*

Little may be said concerning the results obtained with tracer phosphorus, as the few experiments completed show no changes comparable to the oxygen consumption data. However, it should be recognized that these values represent only gross phosphate fractions. Neither specific activity-time relations nor the correspondence between ***in vivo*** and ***in vitro*** P^{32} uptake by this roach muscle was investigated.

Consequently the question of whether or not the increased oxygen consumption

of muscle of allatectomized *Leucophaea maderae* is coupled to phosphorylation, must remain the subject of further research.

The author wishes to express his gratitude to Dr. Berta Scharrer for introducing him into the problems of insect physiology, for donating the animals, both normal and allatectomized, and for working with him in many of the experiments.

Summary

1. Endogenous oxygen consumption of thoracic muscles of *Leucophaea maderae* was no higher in males than in females, but there was a significant increase in this parameter in allatectomized animals of both sexes.

2. Preliminary tracer phosphorus studies were made *in vivo* and the relative specific activities of the muscle fractions are shown.

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FERTILIZATION OF FERTILIZED SEA URCHIN EGGS[1]

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Experiments by Tyler and Schultz (1932) on eggs of the echiuroid worm *Urechis caupo* showed that repeated fertilization was possible. In these experiments development of the eggs was blocked by treatment with slightly acidified sea water within three minutes after the first insemination. When returned to normal sea water at 15 or more minutes later these eggs failed to resume development although containing a spermatozoon. In fact they assumed the appearance of unfertilized eggs. Upon re-insemination a second sperm entered and development proceeded with typical polyspermic cleavage and the formation of abnormal embryos. These experiments have been confirmed on eggs of *Urechis unicinctus* by Kagawa (1952).

In the above-described experiments the possibility of re-fertilization was apparently confined to the first few minutes after fertilization and before the time at which membrane elevation occurs in this species (see Tyler, 1932). More recently it has been shown by Sugiyama (1951) that sea urchin eggs which have been mechanically deprived of their fertilization membrane can be re-fertilized (becoming heavily polyspermic) at considerably later stages provided they are exposed for a short time to Ca- and Mg-free sea water or to isotonic urea. The re-fertilization could be accomplished as late as the two-cell stage. The treatment with Ca- and Mg-free sea water or with isotonic urea usually was for five minutes at a short time after the initial insemination. Sugiyama also reported that if the eggs are put in Ca- and Mg-free sea water before the fertilization membrane rises the latter elevates as a thin structure. When returned to normal sea water two to five minutes later and sperm added the membranes sink to the surface of the egg and re-fertilization occurs.

Hagström and Hagström (1954a) confirmed the re-fertilizability of fertilized sea urchin eggs, using trypsin-treatment of the unfertilized egg to inhibit membrane elevation and Ca- and/or Mg-free sea water treatment of the fertilized eggs to render them susceptible to re-fertilization. In all these experiments the re-inseminated eggs tend to become heavily polyspermic. Evidently the eggs have not only become re-fertilizable as a result of the treatment but also they appear to have lost the ability to propagate the normal rapid block to polyspermy characteristic of the untreated unfertilized egg (see Rothschild, 1954).

Eggs that are activated artificially can also be entered by a number of sperm if the membrane is removed, as in the early experiments of Loeb (1913) and Moore

[1] This investigation was supported by a research grant (C-2302) from the National Cancer Institute of the National Institutes of Health, Public Health Service.

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[3] The work of this author was supported by a grant from the National Science Foundation.

[4] We are indebted to Miss Mary Agnes Jones for technical assistance in this work.

(1916, 1917) or especially if treated with Ca- and Mg-free sea water as in the recent experiments of Ishida and Nakano (1947, 1950) and Nakano (1954).

We have confirmed and extended some of the above experiments. In addition we have found that it is possible to re-fertilize eggs of the sea urchin *Lytechinus pictus* and *L. variegatus* at later stages without any treatment other than removal of the fertilization membrane.

Materials and Methods

Eggs and sperm of the sea urchin *Lytechinus pictus* (Newport Bay, California) and *Lytechinus variegatus* (Alligator Harbor, Florida) were used in most of the experiments. Usually they were obtained by the method of KCl injection (Tyler, 1949). The sand dollar *Dendraster excentricus* was used in two sets of experiments, the eggs and sperm being obtained in the same way.

Mechanical demembranation was accomplished by means of a syringe and 20 gauge needle, the eggs being drawn through the needle at about two minutes after insemination when the fertilization membrane was almost fully elevated.

The trypsin employed in certain of the experiments was a crystalline preparation (Nutritional Biochemicals Corporation) and the papain was a non-crystalline preparation (Merck). The latter was prepared for use as described by Tyler and Spiegel (1956), 2 g. of the papain being extracted with 100 ml. 10^{-3} *M* Versene-sea water and filtered. One ml. of this is added to 100 ml. 0.2% L-cysteine hydrochloride (Merck) in 5×10^{-4} *M* Versene [5]-sea water and adjusted to pH 8.

The Ca- and Mg-free medium (see Tyler, 1953) consisted of a solution of 100 volumes of 0.55 *M* NaCl, 2.2 volumes of 0.55 *M* KCl, 10.3 volumes of 0.37 *M* Na_2SO_4 and ca. 0.6 volumes of 0.55 *M* $NaHCO_3$ to bring the solution to pH 8.

In all of the experiments, except where otherwise noted, the semen was initially diluted to 1% concentration or higher in 10^{-3} molar Versene in sea water and stored as such at the experimental temperature. The Versene was employed because of its ability to maintain the motility and fertilizing capacity of the sperm unchanged for prolonged periods (see Tyler, 1953). The experiments were all run at 17° C., except where otherwise noted.

Results

Re-fertilization of mechanically demembranated eggs after treatment with Ca- and Mg-free sea water

The experiments of Sugiyama (1951) with Ca- and Mg-free sea water treatment could be readily confirmed with eggs of *Lytechinus*. We found, too, that it was not necessary to re-inseminate immediately after return of the eggs to normal sea water. If the demembranated eggs are given a five-minute treatment with Ca- and Mg-free sea water shortly after the first fertilization and returned to normal sea water they retain their ability to be re-fertilized for at least 30 minutes. The results of two experiments of this type are given in Table I.

[5] Ethylenediamine tetraacetic acid. Acknowledgment is made to the Bersworth Chemical Corporation, Framingham, Mass. for a supply of this reagent.

TABLE I

Re-fertilization of eggs of Lytechinus pictus after mechanical demembranation and treatment with Ca- and Mg-free sea water for 5 minutes at 3 minutes after the first insemination

Time between first and second insemination	Percentage of polyspermy (0.1 ml. of 1% semen added per 5 ml. egg suspension)	
	Experiment 1	Experiment 2
Not re-inseminated	4	4
8 minutes	87	49
13 minutes	46	80
23 minutes	54	28
38 minutes	68	12

Re-fertilization of mechanically demembranated eggs without further treatment

As an additional control in the above-mentioned experiments the treatment with Ca- and Mg-free sea water was omitted and a high percentage of polyspermy was obtained upon re-insemination. A series of tests was therefore made of the re-fertilizability of eggs that had been subjected simply to demembranation and re-insemination. The results of five such experiments with eggs of *L. pictus* are listed in Table II. It is evident that fertilized and demembranated eggs can accept additional sperm as late as 40 minutes (at 17° C.) after the first insemination. The re-fertilizability is greater the shorter the time interval between the first and second inseminations, 100 per cent re-fertilization being readily obtained when the re-insemination is done within the first 10 minutes. Even at 15 minutes, as in experiment 5, the eggs can all be re-fertilized if sufficiently concentrated sperm is employed.

The data of the table also show that concentrations of sperm that give 100 per cent re-fertilization cause very little polyspermy when added as initial inseminates to the unfertilized eggs. It also appears from the data of Table II that the process of demembranation permits additional sperm of the initial inseminate to enter the eggs. This effect is, however, relatively slight as a comparison of the percentage polyspermy of the non-demembranated with that of the demembranated eggs in each of the experiments shows. Evidently most of the spermatozoa present in the initial inseminate are not capable of entering the demembranated eggs. Microscopic observation shows that a large proportion of the initially added sperm undergo the spontaneously reversing agglutination characteristic of the reaction with fertilizin in this species of sea urchins (*cf.* Tyler, 1948, 1955), and remain quite active thereafter.

Photographs of re-inseminated demembranated eggs are presented in Figure 1 along with those of non-demembranated and of demembranated and not re-inseminated, controls. In the sample re-inseminated at 10 minutes (Fig. 1e) all of the eggs are heavily polyspermic as shown by the clear areas representing the asters. The polyspermy here is too heavy to permit cleavage. In the various experiments, when the re-insemination is done early (10 to 16 minutes after the initial insemination) and with relatively large amounts of sperm (10^7 or more per ml. of egg suspension) practically all of the eggs show this high degree of polyspermy and failure of cleavage. From observations of the numbers of sperm asters it appears that five or more sperm can enter heavily re-inseminated eggs. In less heavily re-

TABLE II—*Re-fertilization of mechanically demembranated eggs of Lytechinus pictus at various times after fertilization*

Experiment No.	Time between first and second insemination (minutes)	Number of sperm ($\times 10^6$) per ml. of egg suspension		Percentage polyspermy determined at first cleavage (ca. 2 hours)
		First insemination	Second insemination	
1	Not demembranated	1.2	0	1
	Demembranated	1.2	0	3.5
	2½	1.2	1.2	53
	4½	1.2	1.2	51
	10	1.2	1.2	67
	20	1.2	1.2	20
	30	1.2	1.2	13
	40	1.2	1.2	35
2	Not demembranated	28	0	1
	Demembranated	28	0	5.6
	3	28	28	100
	5	28	28	100
	7	28	28	98
	10	28	28	96
	30	28	28	40
3	Not demembranated	2	0	2.5
	Demembranated	2	0	13
	5	2	6	100
	10	2	6	100
	20	2	6	81
	40	2	6	27
	Not dememb. −40	2	0	6
4	Not demembranated	1	0	3
	Demembranated	1	0	22
	10	1	0.4	37
	10	1	1	51
	10	1	4	100
	10	1	8	100
	40	1	0.4	32
	40	1	1	35
	40	1	4	76
	40	1	8	65
5	Not demembranated	49	0	2
	Demembranated	49	0	3
	8	49	49	100
	8	49	245	100
	15	49	49	68
	15	49	245	100
	30	49	49	11
	30	49	245	78
	40	49	49	17
	40	49	245	61

In experiments 1 to 4 the semen was diluted initially to 1% in $10^{-3}M$ Versene in sea water and stored for the later inseminations at that concentration. In experiment 5 the semen was diluted to 10% in ordinary sea water. Sperm counts were made in experiments 2, 4 and 5; sperm estimated in experiments 1 and 3 from dilution on the basis of a content of 2×10^{10} per ml. semen.

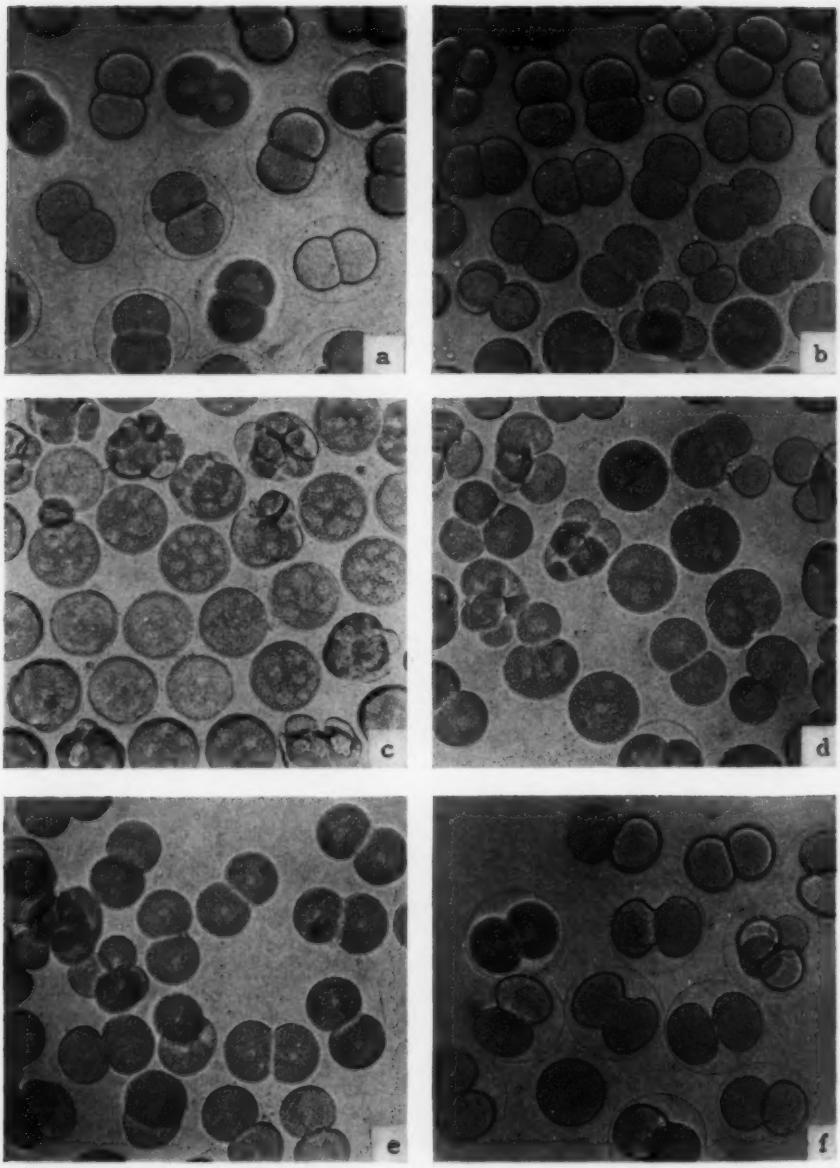

FIGURE 1. Re-fertilization of mechanically demembranated eggs of *Lytechinus pictus*. Insemination number 1 in 0.01%, number 2 in 0.03% semen. Eggs demembranated at one minute

inseminated, demembranated eggs, and in those re-inseminated at later times, the eggs that become re-fertilized generally accept fewer sperm and cleavage occurs in the form characteristic of dispermic or trispermic eggs (see Fig. 1d and e).

Two sets of experiments were performed with mechanically demembranated eggs of the sand dollar *Dendraster excentricus*. In one of these the re-inseminations at 10 and 30 minutes with 10^8 sperm per ml. gave 8.2 and 5.1 per cent polyspermy, respectively, as compared with 1.6 per cent in the non-reinseminated control. In the other experiment re-insemination with 2.2×10^7 sperm per ml. at 6 and 10 minutes gave 9.2 and 7.3 per cent polyspermy and 3.8 per cent in the controls. The results with lower concentrations of sperm and later times of re-insemination differed less significantly from the controls.

Experiments with mechanically demembranated eggs of *Lytechinus variegatus* gave results similar to those obtained with *L. pictus*. Two sets are presented in Table III which includes a comparison of eggs which had been subjected to Ca- and Mg-free sea water with eggs that were not so treated. In the first set the initial insemination was rather heavy (2% of semen which was originally diluted somewhat, perhaps ¼, by fluids shed with the semen). The non-demembranated, once-inseminated controls gave 10.5 per cent polyspermy. Demembranation alone at 1½ minutes resulted in an increase in polyspermy to 45 (Ca- and Mg-free sea water-treated eggs) and 58 per cent (untreated eggs), presumably due to re-fertilization by sperm present in the initial inseminate. Re-insemination at 30 minutes resulted in re-fertilization of most of the eggs when dense sperm suspensions (1% and 0.5%) were used and less with the more dilute suspensions. The treatment with Ca- and Mg-free sea water resulted in generally higher percentages of re-fertilization but the effect in this set is slight.

In the second set of experiments listed in Table III the initial insemination was performed with more dilute sperm and the control polyspermy was lower (7%). Demembranation without reinsemination resulted in a small increase in percentage polyspermy (17% for the Ca- and Mg-free sea water-treated eggs and 13% for the untreated eggs). Upon re-insemination at 36 minutes the large increase in polyspermy was again obtained when relatively dense sperm suspensions were used and significant increases even in the dilute suspensions. Again the Ca- and Mg-free sea water-treated eggs give somewhat more re-fertilization than the untreated eggs. In this set inseminations of normal, unfertilized, eggs were made, at the same time and with the same sperm suspensions that were employed for the re-insemination. These controls (not listed in the table) gave 100 per cent fertilization in all but the 0.0063 per cent concentration of sperm (semen) which gave 90 to 95 per cent. Versene was not used in these experiments on *L. variegatus*.

Re-fertilization of trypsin-treated eggs

When unfertilized sea urchin eggs are treated with trypsin they fail to elevate a membrane upon fertilization as Runnström *et al.* (1943) originally showed. Hagström and Hagström (1954a) found that such eggs could be re-fertilized if

after initial insemination. All pictures taken at time of first cleavage of control eggs. Magnification 95 ×. (a) Control, non-demembranated eggs. (b) Control, demembranated eggs. Re-inseminated (c) at 10 minutes, (d) at 20 minutes, (e) at 40 minutes after first insemination. (f) Control eggs, singly inseminated in 0.03% semen, at 40 minutes after beginning of experiment.

TABLE III

Re-fertilization of mechanically demembranated eggs of Lytechinus variegatus with and without treatment with Ca- and Mg-free sea water; at 22° C.

Concentration of sperm (semen)		Percentage polyspermy	
First insemination, %	Second insemination at 30 minutes	Eggs exposed to Ca- and Mg-free sea water at 6 to 12½ minutes	Eggs washed in sea water at corresponding times
2	Not demembranated Not re-inseminated		10.5
2	Demembranated but not re-inseminated	45	58
2	1%	99.5	97
2	0.5	97.5	91
2	0.25	90	83.5
2	0.125	93	77.5
2	0.063	72.5	73.5
	Second insemination at 36 minutes	Ca- and Mg-free sea water at 5 to 11 minutes	
0.1	Not demembranated Not re-inseminated		7
0.1	Demembranated but not re-inseminated	17	13
0.1	0.1	74	86
0.1	0.05	77.5	56
0.1	0.025	60	52
0.1	0.0125	51	39
0.1	0.0063	44	40

TABLE IV

Re-fertilization of trypsin-treated eggs of Lytechinus pictus

First insemination; after 10 minute treatment of eggs with 0.01% trypsin in sea water and washing; Sperm per ml.	Second insemination		Percentage polyspermy
	Time of insemination in minutes	Sperm ($\times 10^6$) per ml.	
4×10^6	—	0	12
	5	8	47
	30	8	48
8×10^6	—	0	19
	5	8	73
	30	8	39
16×10^6	—	0	26
	5	8	76
	30	8	53

they were exposed to Ca- or Mg-free sea water for a short time at various times after the first fertilization. Practically no re-fertilization was obtained without the treatment with Ca- or Mg-free sea water.

We have found that when unfertilized eggs of *Lytechinus pictus* are exposed to trypsin, washed and inseminated, they can later be re-fertilized, without further treatment other than re-insemination. Table IV presents the results of a set of

TABLE V

Re-fertilization of papain-treated eggs of Lytechinus pictus

First insemination; eggs added to papain solution containing sperm; sperm per ml.	Time of papain treatment (minutes)	Second insemination (after washing eggs with sea water)		Percentage polyspermy
		Time in minutes	Sperm ($\times 10^6$) per ml.	
2×10^6	6	—	0	1
	6	14	2	2
	6	14	10	75
	6	40	2	2
	6	40	10	2
1.5×10^6	11	—	0	3
	11	18	1.5	10
	11	18	6	85
	11	40	1.5	8
	11	40	6	55
3×10^7	5	—	0	3
	5	7	30	95
	5	7	120	100
	10	—	0	5
	10	11	30	100
	10	11	120	100
	15	—	0	5
	15	17	30	98
	15	17	120	100
	20	—	0	18
	20	21	30	26
	20	21	120	100
	30	—	0	18
	30	32	30	100
	30	32	120	100
	40	—	0	17
	40	42	30	98
	40	42	120	100

experiments in which the eggs were treated for ten minutes with a solution of 0.01 per cent trypsin in sea water. After washing, three samples were inseminated, each with a different concentration of sperm (4×10^6, 8×10^6 and 16×10^6 per ml.). Samples from each of these were re-inseminated at 5 minutes and at 30 minutes. Trypsin-treatment is known (see Hagström and Hagström, 1954b; Tyler and Metz, 1955) to render sea urchin eggs susceptible to polyspermy. In the present experiment this is evident in the relatively high percentage (12, 19 and 26) of polyspermy

obtained without re-insemination. However, the data of the table show that upon re-insemination re-fertilization occurs as late as 30 minutes after initial fertilization. The re-insemination at 5 minutes is relatively more effective.

Re-fertilization of papain-treated eggs

Treatment of unfertilized sea urchin eggs with papain also destroys their ability to elevate a membrane upon fertilization. The fertilizability of the papain-treated eggs is, however, often rather poor. Tyler and Spiegel (1956) showed that fertilization proceeds very well if the eggs are added to a suspension of sperm in the papain solution. In these circumstances a rather unique phenomenon is observed; namely, a fertilization membrane is elevated, as fully as in normal sea water and then, within the next three or four minutes, retracts to the surface of the egg from which it is not distinguishable. Such eggs, even when soon removed from the papain solution, show no signs of a fertilization membrane at the time of cleavage.

In the present experiments *Lytechinus* eggs were added to sperm in papain solution. At various times after retraction of the fertilization membrane to the surface the eggs were washed in sea water and re-inseminated. The results of three sets of experiments are presented in Table V. The insemination in the papain solution results in a relatively low (1 to 5) percentage of polyspermy except in those experiments (in the third set) where the eggs were allowed to remain in the solution for 20 minutes or longer in which case it rose somewhat (17 to 18%). Upon re-insemination one hundred per cent polyspermy was obtained in many of the tests. As in the experiments with the mechanically demembranated eggs and the trypsin-treated eggs the re-fertilizability is higher the sooner the second insemination is done and the greater the concentration of sperm. This statement applies to comparisons within a set of experiments since in experiments performed on different days the absolute values may differ, as a comparison of the first two sets of experiments in Table IV indicates. If the eggs are allowed to remain in the papain solution until shortly before the re-insemination, as in the third set of experiments listed in Table IV, the re-fertilizability at the later times remains high. As in the mechanically demembranated eggs the degree of polyspermy following re-insemination is often too high to permit cleavage, the polyspermic nature of the eggs being indicated by the many asters present.

Discussion

It has been shown in the present experiments that fertilized eggs that have been removed from their fertilization membrane can be re-fertilized. This is contrary to the generally accepted views and the experience of many investigators (see Lillie, 1919; Just, 1939), the surface of the membrane-less fertilized egg being considered unreceptive to sperm. While the experiments of Tyler and Schultz (1932) demonstrated re-fertilizability of fertilized eggs this was confined to the period before membrane elevation occurs. The experiments of Sugiyama (1951) and of Hagström and Hagström (1954a, 1954b) showed that re-fertilization was possible at later stages, provided that the membrane-less eggs were exposed for a while to Ca- and/or Mg-free media. Without such treatment these workers obtained no significant re-fertilization of membrane-less eggs upon re-insemination. The dif-

ference in results may in part be due to differences in the species of echinoid used. In the present experiments the two preliminary experiments with *Dendraster* showed relatively little re-fertilizability in comparison with the results with *Lytechinus*. The difference may also be due in part to differences in the concentration of sperm used for re-insemination.

Sugiyama (1951) does not state the concentration of sperm used in his test (p. 336) of the re-fertilizability of denuded, untreated eggs of *Strongylocentrotus*. If it were 10^{-2} (one per cent semen; probably about 2×10^{-8} sperm per ml.), which, as shown in his Table I, was used for the eggs treated with Ca- and Mg-free sea water, this would be higher than concentrations found in the present experiments with *Lytechinus* to be capable of re-fertilizing practically all of the demembranated, untreated, eggs. Hagström and Hagström (1954a, 1954b) used 2×10^{6} sperm per ml. for re-inseminations of eggs of *Paracentrotus lividus* without getting significant re-fertilization of the controls. This is within the effective range for *Lytechinus* when the re-inseminations are performed early. It would appear, then, that species differences were largely responsible for the difference in results.

The use of Versene in the sperm suspension may have contributed to the effectiveness of the sperm in re-fertilization, by virtue of its favorable action (Tyler, 1953) on the fertilizing capacity of the sperm. However, it is not a determining factor in the present results, as the experiments (see Table II) in which it was omitted show. Also, it should be noted that in those experiments in which it was employed in the sperm suspension the amounts added with the sperm to the eggs were such as to make the concentration less than 10^{-5} molar.

One feature of *Lytechinus* eggs that might be related to the apparent ease with which they can be re-fertilized is the relative thinness, and slowness of formation, of the ectoplasmic (hyaline) layer. However, this layer is distinctly visible, and well formed, by about 15 minutes after fertilization, whereas re-fertilization is possible after 40 or more minutes.

It has been noted in the presentation of the data that the re-inseminated eggs tend to become highly polyspermic. This occurs with concentrations of sperm which, when used as a first inseminate, result in very little or no polyspermy. Evidently the demembranated fertilized egg does not possess the capability of the unfertilized egg of excluding all but one sperm. Experiments by Rothschild and Swann (1949, 1950, 1951, 1952; reviewed by Rothschild, 1954) have shown that the response of the unfertilized egg is characterized by a rapid change that passes over the surface in about two seconds, from the point of the entering sperm, that renders the surface about 95 per cent unreceptive to other sperm. This is followed by a change, requiring about 60 seconds, that makes the surface completely resistant. These changes are in advance of elevation of the fertilization membrane. However, the surface of the fertilization membrane is presumably this altered surface of the freshly fertilized egg. The surface of the denuded fertilized egg has evidently not become resistant to sperm and also lacks the ability to propagate a fast block to the entrance of more than one spermatozoon.

The ability of the denuded fertilized egg to be fertilized would imply that its surface possesses a sperm receptor substance similar to that of the unfertilized egg. In the latter this is attributed (see Tyler, 1948, 1955) to the substance known as fertilizin. It would be of interest to learn then whether the same kind of substance

is present on the surface of the denuded unfertilized egg. Experiments by Motomura (1950, 1953a, 1953b) have provided some evidence for the existence of a cytofertilizin within the egg. The evidence also points to the ectoplasmic (hyaline) layer of the fertilized egg as being the likely source. If the results are not due to possible incomplete removal of the fertilizin of the unfertilized egg, they provide then an interpretation of the re-fertilizability of the fertilized egg on the same basis as that of initial fertilization; namely that the specific adherence of the sperm is due to reaction of its anti-fertilizin with fertilizin of the egg. If the fertilizin-like substance is assumed to be present in lower concentration on the surface of the denuded fertilized egg than on that of the unfertilized egg, or if it is assumed to be less reactive, this would help explain why high concentrations of sperm are required for re-fertilization. Further, it would appear that this postulated fertilizin-like substance is only slowly lost or altered on the surface of the denuded fertilized egg, the egg being re-fertilizable for a considerable period. Since brief treatment with Ca- and/or Mg-free media permits re-fertilization at still later stages and such treatment partially dissolves the ectoplasmic (hyaline) layer it would further appear that the treatment exposes additional fertilizin-like material. Tests of these and related possibilities will be the subject of future investigations.

Summary

Fertilized, mechanically demembranated, eggs of the sea urchins *Lytechinus pictus* and *Lytechinus variegatus* can be re-fertilized when heavily re-inseminated at a half-hour or more (17° to 22° C.) after the initial fertilization. The re-fertilization occurs more readily the earlier the second insemination is performed. The re-inseminated eggs tend to become heavily polyspermic. Concentrations of sperm that give 100 per cent re-fertilization do not cause any significant polyspermy when used as a first inseminate on normal unfertilized eggs. The results show not only that the demembranated fertilized egg is capable of accepting additional sperm but also that it lacks the ability to propagate a fast block to all but one sperm. Treatment of the unfertilized eggs with trypsin, so as to suppress membrane formation, or with papain at the time of fertilization, so as to cause reversal of membrane elevation, also permits re-fertilization upon re-insemination without further treatment. The results are interpreted in terms of the possible presence of fertilizin-like sperm receptors on the surface of the denuded fertilized egg.

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ELEVATION AND RETRACTION OF THE FERTILIZATION MEMBRANE OF ECHINODERM EGGS FERTILIZED IN PAPAIN SOLUTIONS[1]

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It has been shown by Runnström *et al.*, (1943), Moore (1949) and others that treatment of unfertilized eggs of sea urchins with solutions of trypsin or chymotrypsin causes the eggs to fail to elevate a membrane upon fertilization. Normal development can ensue after such treatment, although the quantity of sperm required for successful fertilization is found (Tyler and Metz, 1956) to be greater than for untreated eggs. The treated eggs are also more susceptible to polyspermy (Hagström and Hagström, 1954) and more susceptible to cross-fertilization (Hultin, 1948; Bohus-Jensen, 1953; Tyler and Metz, 1956).

The action of trypsin in causing failure of membrane-elevation upon fertilization has been interpreted (Runnström *et al.*, 1943; Minganti, 1953) as due to a dissolution of the vitelline membrane of the unfertilized egg, which, together with material of exploded cortical granules, is considered (Motomura, 1941; Runnström *et al.*, 1944) to be the precursor of the fertilization membrane. Doubts have, however, been expressed (Mitchison and Swann, 1952; Hillier *et al.*, 1952) as to the separate existence of a vitelline membrane and of its dissolution by proteolytic enzymes (see also Tyler and Metz, 1956). The examination of the egg surface by polarization-microscopy (Swann and Mitchison, 1951; Mitchison and Swann, 1952) and by electron microscopy (Hillier *et al.*, 1952; see, however, McCulloch, 1952) revealed no distinct structure corresponding to the vitelline membrane. Runnström *et al.* (1943) showed that trypsin-treated eggs behave differently from untreated eggs upon shrinking and swelling in hypertonic and hypotonic solutions, which could be interpreted as due to loss of the vitelline membrane. It is possible, however, that the difference might be due to an alteration of the surface by combination with trypsin rather than the removal of anything therefrom. Parpart (personal communication) examined trypsin-treated eggs of *Arbacia* under the "television microscope" and could observe no change in the appearance of the vitelline membrane.

There are also cases, notably in the sand dollar *Dendraster excentricus* (Moore, 1951) and in the heart urchin *Echinocardium cordatum* (Runnström, 1948), in which treatment of the unfertilized egg with trypsin causes an elevation of a membrane.

In the present experiment the action of the enzyme papain was examined during the process of fertilization and it was observed that membrane elevation could occur

[1] This work was supported by a grant (C-2302) from the National Cancer Institute of the National Institutes of Health, Public Health Service.

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in the presence of the enzyme but that the membrane subsequently returned to the surface of the egg.

Material and Methods

Eggs of the sea urchins *Strongylocentrotus purpuratus* and *Lytechinus pictus* and of the starfish *Asterias forbesii* were used in this work.

The papain solution was prepared by extracting for several hours in the cold two grams of papain (Nutritional Biochemical Corporation) with 100 ml. of sea water containing 10^{-4} moles of Versene [3] (ethylenediaminetetraacetic acid). The Versene was included because of its effect of improving the fertilizing capacity of the sperm (Tyler, 1953). In some experiments the Versene was omitted. The extract (termed a 2% solution) was filtered and diluted (with 10^{-3} *M* Versene or with sea water) to the concentration desired. To this, just before use, cysteine hydrochloride was added to give a concentration of 0.2% and the pH adjusted to between 8.1 and 8.2.

Results

When unfertilized eggs of *Strongylocentrotus* or of *Lytechinus* were treated with solutions of papain ranging from 0.01 to 2% for periods of 5 seconds to one hour, and then inseminated in the solution, fertilization ensued without the elevation of a fertilization membrane. Such eggs cleaved while in the papain solution, the blastomeres separating from one another. When the unfertilized eggs were washed with sea water after treatment with papain solution (0.01% to 2% for periods of five seconds to one hour) they similarly failed to elevate a membrane upon fertilization. Cleavage of such eggs was normal and normal plutei developed.

When unfertilized eggs were added to a suspension of sperm in a 0.04% or 0.02% solution of papain, membrane elevation occurred. However, the membrane subsequently retracted to the surface of the egg. Figure 1 illustrates the course of this phenomenon in eggs of *Strongylocentrotus*. In this series eggs were added to a 0.1% suspension of semen in a 0.04% solution of papain. The membrane is observed to be fully raised after 1½ minutes (Fig. 1c). From then on it progressively shrinks until at 5½ minutes it has returned to the egg's surface, from which it is no longer distinguishable. If left in the solution the blastomeres tend to separate upon cleavage. If removed from the solution at 5½ to 20 minutes cleavage is normal with a distinct ectoplasmic (hyaline) layer visible, but no indication of a fertilization membrane (see Fig. 1, 1). One hundred per cent normal cleavage can be obtained in this manner and later development is as good as in the control eggs.

Similar results were obtained with the eggs of *Lytechinus* and those of *Asterias*. In the latter the membrane elevates completely at 5 minutes and retracts to the surface (no longer distinctly visible) at 20–30 minutes after insemination.

Discussion

The main point of the present paper is that a fertilization membrane can be elevated and then retracted, as described above, during the first few minutes after insemination. There is no indication of the presence of this membrane as a dis-

[3] Supplied by Bersworth Chemical Corporation, Framingham, Mass.

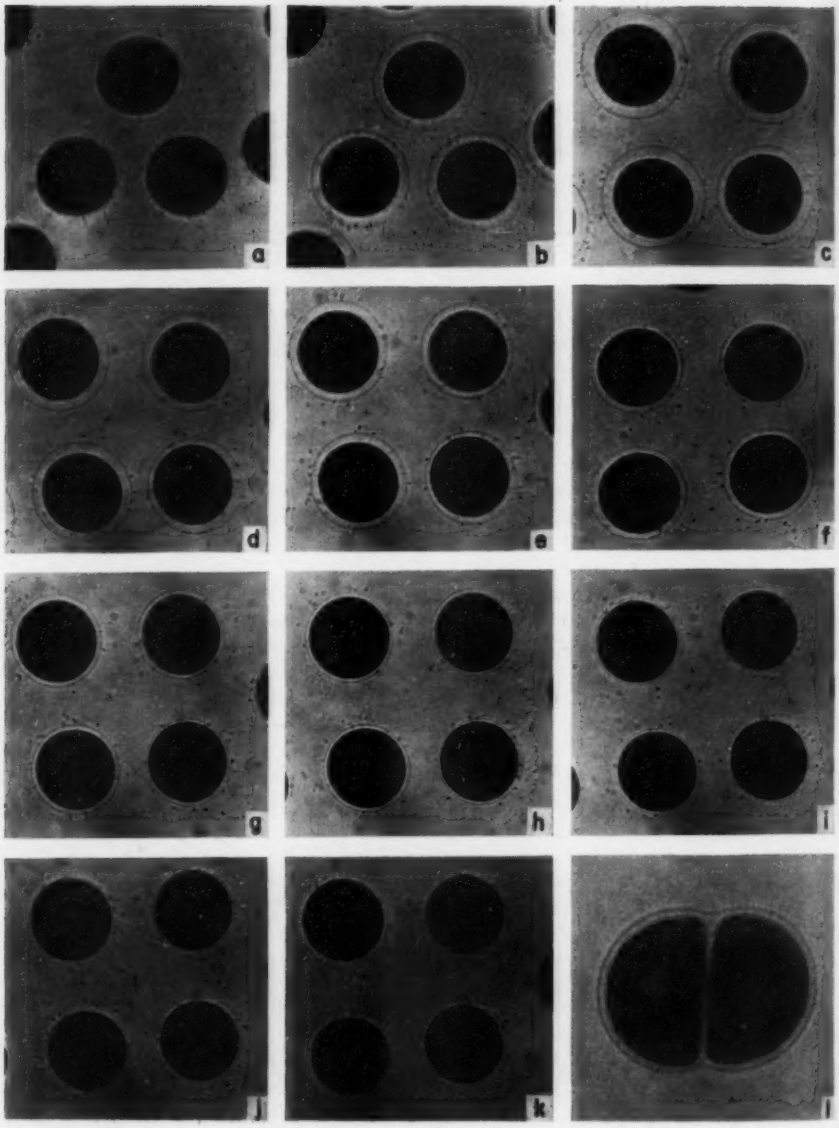

FIGURE 1. Papain demembranation of *Strongylocentrotus purpuratus* eggs. One drop sperm (in 0.02% papain) mixed with one drop egg suspension on slide sealed with vaseline. Figure (a) taken 35 seconds after eggs added, figures (b)–(k) taken at 30-second intervals. Figure 1 (l) shows a papain-treated egg (removed to sea water at 15 minutes) in the 2-cell stage. Magnification: Figures (a)–(k), 147 ×; figure (l), 333 ×.

tinct entity during subsequent development of the egg. This may be interpreted to mean that further enzyme-action after the membrane has reached the egg's surface results in its dissolution. It should be noted, however, that when eggs of sea urchins are treated with proteolytic enzymes after the fertilization membrane has fully formed (a few minutes after fertilization) the latter is not visibly affected (see Moore, 1949; Runnström *et al.*, 1943; Minganti, 1953). The same is true for treatment with papain solutions. This, then, does not support the interpretation that there has been a dissolution of the membrane by enzymatic action after return to the surface. Our present interpretation is that the contracted membrane fuses with the egg surface and its material remains a part thereof during subsequent development.

Summary

When eggs of sea urchins and starfish are added to a suspension of sperm in papain solutions (0.02 to 0.04%), a fertilization membrane is elevated in the normal time and, after reaching full separation, retracts to the surface of the egg. This process is completed in approximately 5 minutes in the case of sea urchins (*Strongylocentrotus purpuratus* and *Lytechinus pictus*) and 25 minutes in the case of starfish (*Asterias forbesii*). It is not visible during subsequent development after removal of the eggs from the solution, and development to late stages is normal. Treatment of unfertilized eggs with papain prevents membrane elevation upon fertilization, as reported for other proteolytic enzymes. Treatment of eggs with fully formed fertilization membranes shows no effect on these. It is suggested that the retracted membrane, obtained by fertilization in papain solution, fuses with the egg surface, and that, in general, the question remains open as to whether or not proteolytic enzymes dissolve the vitelline membrane of the unfertilized egg.

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PHYSIOLOGY OF INSECT DIAPAUSE. X. AN ENDOCRINE MECHANISM FOR THE INFLUENCE OF TEMPERATURE ON THE DIAPAUSING PUPA OF THE CECROPIA SILKWORM

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Among the fifteen thousand Cecropia silkworms which we have reared during the past ten years, only an occasional individual has failed to begin a prolonged period of diapause immediately after pupation. Many of the animals underwent pupation in early summer, at the season when one might expect environmental temperature and photoperiod to favor uninterrupted growth and metamorphosis. Yet the intervention of diapause was no less uniform than in animals pupating in September or October. The Cecropia silkworm is therefore a typical single-brooded or "univoltine" insect.

The pupal diapause provides for the overwintering of the species. Though the pupa undergoes little or no morphological advance during this period, the months of exposure to the low temperatures of winter are not "time out." By its action within the diapausing insect, low temperature promotes certain physiological changes which assure the initiation of adult development the following spring. The detailed nature of these changes has been the central concern of the present study.

The investigation has been greatly assisted by a series of recent reviews surveying the voluminous literature pertaining to insect diapause (Andrewartha, 1952; Andrewartha and Birch, 1954; Lees, 1955). These reviews, to which the reader is referred for background to the present study, make clear that the potentiation of development by low temperature is a distinguishing feature of diapause, serving to differentiate diapause from hibernation, quiescence, and other forms of dormancy which are enforced by low temperature.

Materials and Methods

The investigation was performed on pupae of the Cecropia silkworm (*Platysamia cecropia* L.). The larval stages were reared under large nylon nets on wild-cherry trees. The cocoons were harvested immediately after spinning and placed at 20 to 25° C. for at least six weeks prior to use. Pupation occurred during the

[1] The study was aided by a grant from the U. S. Public Health Service and an Institutional Grant to Harvard University from the American Cancer Society. The present report was prepared during the tenure of a John Simon Guggenheim Memorial Foundation Fellowship at the Department of Zoology, Cambridge University. To members of this Department, and especially to Professor V. B. Wigglesworth, F.R.S., and Dr. Tony Lees, the author is grateful for numerous helpful discussions of the experimental results and the manuscript itself. The latter was also read and improved following certain suggestions of Drs. Berta Scharrer and William Van der Kloot.

first two weeks and was followed by the characteristic decline in metabolic rate to the extremely low diapausing level (Schneiderman and Williams, 1953).

The temperature relations of several other species of diapausing saturniids have also been studied in detail. However, in the present report attention centers on the Cecropia silkworm, and only one series of experiments will be described on the related species, *Samia walkeri* (*cynthia*).

Ten years ago, at the outset of the present study, it was already evident that a quantitative approach to the problem required the use of a rather large number of individuals in testing each condition and circumstance. This practice, in turn, complicated the management of the experimental findings, since the latter sometimes showed considerable scatter. The procedure which was finally adopted and applied throughout the present study was to define the mean of each distribution, as well as the latter's standard deviation, by the use of probability paper (Codex

TABLE I

Previously unchilled Cecropia pupae at each of a series of constant temperatures: time for initiation of development and for adult development itself

Temp.	No. animals	Weeks to initiate development		Weeks to complete development		Total Weeks
		Mean ± S.D.	Recip. × 100	Mean ± S.D.	Recip. × 100	
2½° C.	20	∝	0	∝	0	∝
6	20	ca. 41	2.6	∝	0	∝
10	18	25.0 ± 1.9	4.0	16.3 ± 2.0*	6.1	41.3
15	19	23.3 ± 3.9	4.3	11.9 ± 1.4	8.4	35.2
20	20	37.0 ± 9.4	2.7	4.9 ± 0.6	20.4	41.9
25	20	25.4 ± 4.9	3.9	3.1 ± 0.9	32.3	28.5

* Adult moths with extremely dark pigment.

Book Co., Norwood, Massachusetts, No. 32,451, Normal Ruling). The application of this technique, as well as various other experimental procedures, will be described in the sections that follow.

RESULTS

1. *Unchilled pupae at specific constant temperatures*

A. Initiation of adult development

When unchilled Cecropia pupae are placed at a particular temperature within the range 6 to 35° C. and maintained thereafter at this same constant temperature, the pupal diapause is ultimately terminated and adult development begins. In no case, however, does this occur immediately. Irrespective of what the temperature may be, adult development, as signalled by the retraction of the epidermis from the overlying pupal cuticle, is initiated only after prolonged exposure to the constant temperature. Prior to the actual initiation of adult development, even a detailed dissection of the pupa fails to reveal any clear-cut change from its condition at the outset.

In order to appraise the matter in quantitative terms, a study was made of the

time required for the initiation of adult development at each of a series of constant temperatures. For this purpose, a homogeneous batch of animals (which had pupated 6 to 8 weeks earlier and has subsequently been stored at 20–25° C.) was removed from cocoons, placed in individual capped "creamers," and distributed among seven temperature cabinets or constant temperature rooms at temperatures ranging from 2½ to 30° C. Thereafter at weekly intervals, each pupa was checked for the initiation of adult development, as judged by the retraction of the leg epidermis.

In the experimental results summarized in Table I, it will be observed that a considerable period was required for the initiation of adult development at all of

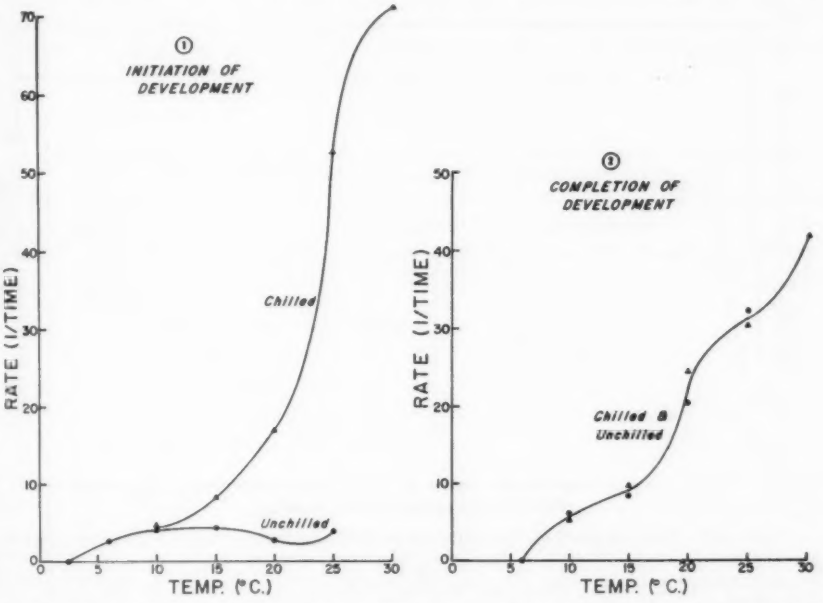

FIGURE 1. The rate (100/Weeks) of initiation of adult development at each of a series of constant temperatures. The lower curve describes the response of previously unchilled pupae; the upper curve, the response of pupae which had been chilled for 15 weeks at 6° C. prior to the experiment. The period of preliminary chilling greatly accelerates the initiation of development.

FIGURE 2. The rate (100/Weeks) of adult development at a series of constant temperatures after development has begun. The response of previously unchilled pupae (circles) cannot be distinguished from that of previously chilled pupae (triangles).

the constant temperatures within the range 2½ to 30° C. Indeed, at 2½° C. adult development never began, and pupae at this temperature invariably died after about a year.

In the lower curve in Figure 1 the results have been computed and plotted on a rate (1/Time) basis. The relatively minor effects of temperature are here clearly evident. Even these minor effects are paradoxical in that the rates encountered at 20° and 25° C. are actually lower than those at 10° and 15° C.

B. Completion of adult development

Each animal in the experiment just considered was permitted to continue to develop and to emerge as an adult moth. In this manner it was possible to ascertain the effects of temperature on the kinetics of adult development itself. The results are recorded in Table I, and plotted on a rate (1/Time) basis in Figure 2.

It is clear that once adult development has begun, a striking change occurs in the insect's response to environmental temperature. Temperature now becomes a prime determinant of the rate of adult development—a fact which is signalled in Figure 2 by the curve's pronounced and positive slope.

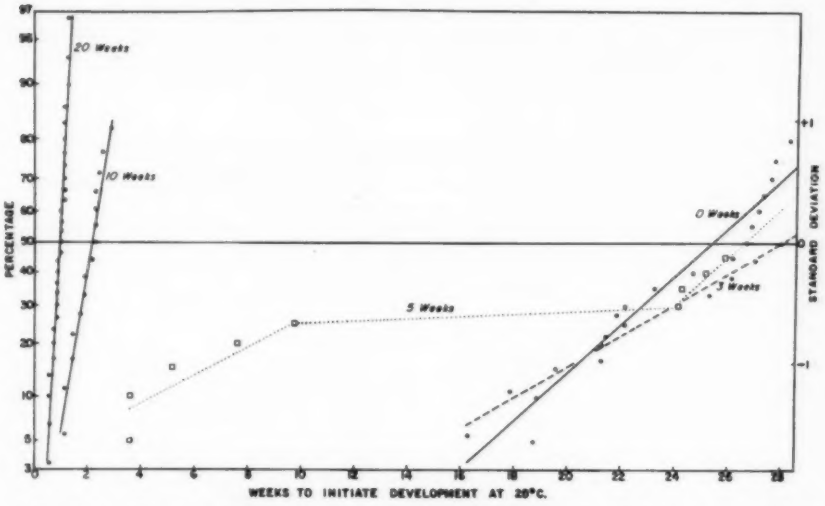

FIGURE 3. Time required for the initiation of adult development at 25° C. after preliminary exposure to 6° C. for 0–20 weeks. By the use of a probit scale on the Y-axis, each distribution is converted into a straight line. The discontinuous line for the 5-week series signals a bimodal distribution.

Though the formation of the adult moth, as noted in the preceding section, ultimately begins after about ten months at 6° C., such animals never proceed beyond early development and ultimately die before reaching a stage corresponding to that which would be attained at 25° C. after only three days. At the slightly higher temperature of 10° C., approximately 3½ months elapse between the initiation and completion of development. Adult moths developing at this constant temperature are extremely dark, the reddish pigments of scales and hairs being replaced by black and the white pigments by gray. At 10° C. the insect is incapable of sufficient muscular effort to emerge spontaneously from the pupal exuviae and ordinarily does not do so unless returned to higher temperatures. By contrast, at 15° C. and all higher temperatures up to 30° C., the moths show normal pigmentation and are able to emerge spontaneously and spread their wings. At the still higher temperature of 35° C., development proceeds with considerable rapidity, but

the moths are extremely pale and feeble, and female individuals show few if any eggs.

2. Previously chilled pupae at specific constant temperatures

The experiment described in the preceding section was repeated on a homogeneous batch of pupae which had been previously exposed to 6° C. for 15 weeks. The results are summarized in Table II and plotted on a rate (1/Time) basis in Figures 1 and 2.

The upper curve in Figure 1 makes clear the extraordinary difference in the temperature response of chilled pupae from that previously defined for unchilled pupae (lower curve). Temperatures above 10° C. now greatly accelerate the initiation of adult development. For example, at 25° C. the time required for the initiation of adult development is decreased from 25 weeks to 13 days.

TABLE II

Previously chilled pupae (6° C. for 15 weeks) at each of a series of constant temperatures: time for initiation of development and for adult development itself

Temp.	No. animals	Weeks to initiate development*		Weeks to complete development		Total Weeks
		Mean ± S.D.	Recip. × 100	Mean ± S.D.	Recip. × 100	
10° C.	8	21.4 ± 7.4	4.7	19.1 ± 4.3	5.2	40.5
15	19	12.1 ± 14.1	8.3	10.3 ± 0.9	9.7	22.4
20	19	5.9 ± 4.7	17.0	4.1 ± 1.9	24.4	10.0
25	30	1.9 ± 1.0	52.7	3.3 ± 0.3	30.3	5.2
30	19	1.4 ± 1.0	71.4	2.4 ± 0.3	41.7	3.8

* Not counting the previous 15 weeks at 6° C.

Attention is directed to the tempo of adult development after the latter has begun (Fig. 2). Manifestly, the curve descriptive of the response of previously chilled pupae cannot be distinguished from that recorded for unchilled pupae. We are therefore led to infer that the pupal diapause, as a physiological and biochemical phenomenon, is primarily concerned with events which culminate in the initiation of development, and that it is little concerned with adult development itself. Our present problem, in brief, is to account for the differences between the upper and lower curves in Figure 1.

One can say immediately that the upper curve, which describes the response of previously chilled pupae, is not remarkable: it is a familiar experience to find biological processes accelerated by temperature. The previously chilled pupa, in effect, is responding to temperature like a normal non-diapausing insect. But what is remarkable is the effective "insulation" of the previously unchilled pupa from the accelerating action of temperature. An explanation of this effect was the primary objective of the present study.

3. Graded exposure of unchilled pupae to 6° C.

We have already considered and illustrated in Figure 1 the way in which preliminary exposure to 6° C. greatly decreases the time required for the initiation of

development at 25° C. The speed with which the response ensues at 25° C. becomes a measure of the persistent effects of the previous exposure to low temperature. In the experiment recorded in Figure 1, the exposure to 6° C. was for 15 weeks. However, the same technique obviously can be used to appraise the quantitative effects of any other duration of chilling. This maneuver has been used extensively during the present study in order to probe the latent effects of low temperature.

Figure 3 summarizes a part of the results of a detailed study of this type in which the effects of the duration of exposure to 6° C. were studied. The experiment was performed on 160 Cecropia pupae which were harvested in freshly spun cocoons on August 7–11, 1953, and temporarily stored at 25° C. for ten weeks prior to the experiment. The cocoons were then placed in capped cardboard containers and stored at 6° C. Thereafter, at regular intervals ranging from 0 to 45 weeks a group of twenty pupae were removed from cocoons, placed in individual, uncapped "creamers," and returned to 25° C. The time which each animal required to initiate adult development was then ascertained in terms of the initiation of retraction of the leg epithelium—a signal of the second day of adult development at 25° C.

The effects of the graded exposures to 6° C. are plotted in Figure 3 on a probability grid, whose use was mentioned in the section on Methods. This type of plot has the advantage of transforming normal distributions into straight lines; it likewise permits one to define the mean and the standard deviation with far greater precision than is possible by any simple arithmetic procedure. In Figure 3 a straight line has been drawn by inspection through the distribution of responses after each preliminary exposure to 6° C. It is immediately apparent that 3 weeks at 6° C. failed to facilitate the subsequent response at 25° C.; indeed, the mean (28 weeks) was slightly prolonged beyond the value after no chilling (25.4 weeks); moreover, as signalled by the flatter slope to the curve, the scatter in the data (which was already large after no chilling) was further enhanced after three weeks at 6° C.

In Figure 3 attention is now directed to the distribution of responses at 25° C. after preliminary exposure to 6° C. for 10 and 20 weeks, respectively. It is clear that all these data fall nicely into straight lines whose steep slopes signify minor degrees of scatter. It is of particular interest and importance to note that the means, thus defined, are extremely small and range from 7 to 15 days. Consequently, a spectacular effect of the low temperature treatment has taken place between the third and tenth weeks at 6° C.

Within this critical period the response was examined after 5 weeks of chilling. In the results recorded in Figure 3, it is necessary to place a discontinuous line through the data thus obtained. This signifies that the distribution of responses is bimodal. Five of the twenty pupae, irrespective of sex, showed a significant effect of the low temperature treatment. An extrapolation to the 50 per cent level of a line joining these five points suggests a mean value of approximately 16 weeks for the initiation of development. But for the next five animals to initiate development, it is clear that 5 weeks at 6° C. was without detectable effect, the estimated mean being, in fact, slightly greater than that observed after no chilling.

The response after 5 weeks of chilling is therefore of particular interest in that 25 per cent of the pupae showed a significant influence of the preliminary low

temperature treatment while 75 per cent did not. Since the response after 10 weeks of chilling is homogeneous, it follows that in the normal course of events, this residual 75 per cent must shift from the delayed to the accelerated type of response between the fifth and tenth weeks at 6° C.

A point of considerable theoretical interest is the lack of overlap between any regions of the 5-week and 10-week curves in Figure 3. This signifies that even in the five animals which responded most promptly in the 5-week series, the physio-

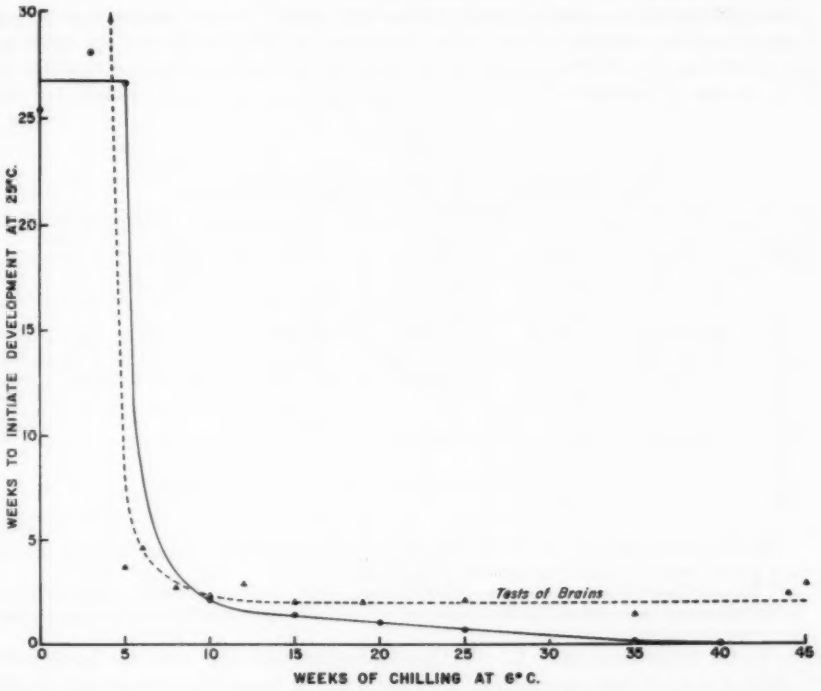

FIGURE 4. The unbroken curve records the time required for the initiation of development at 25° C. as a function of the duration of preliminary exposure to 6° C. The broken line records the corresponding changes occurring in the endocrine activity of the pupal brain. For explanation, see text.

logical changes responsible for the accelerated response had not proceeded to completion by the fifth week, but, so to say, were caught "in transit."

On the basis of the results just considered, one can therefore recognize a sequence of two relatively stable states during pupal life, corresponding, respectively, to the *delayed* and the *accelerated* responses to the warm temperature. Moreover, the transition from the first to the second state shows the kinetics of a threshold reaction, being initiated by each individual during an apparently brief period after the third week at 6° C. and prior to the tenth week. It is equally clear that this

reaction, which then effects the transition, can scarcely be "all or none," since it requires at least several weeks to pursue its full course.

These various conclusions are illustrated in Figure 4 where the mean times required for the initiation of development at 25° C. have been read from Figure 3 and plotted as a function of the duration of preliminary chilling at 6° C. The abrupt descending limb of the curve has been placed just after the fifth week at 6° C., though, for reasons mentioned above, its true position for about one-fourth of individuals is slightly to the left of this point.

Notwithstanding persistent uncertainties with respect to the precise time-course of the descending segment of the curve, there can be little doubt that the relationship is dominated by the transition of the pupa from a state of delayed response at 25° C. to one of accelerated response. This transition, we have inferred, is com-

TABLE III

Time for initiation of development of Cecropia pupae at 25° C. after indicated exposures to specific constant temperatures*

Exposure (weeks)	2½° C. (weeks)	6° C. (weeks)	10° C. (weeks)	15° C. (weeks)	20° C. (weeks)	30° C. (weeks)
0	25.4 ± 4.9	25.4 ± 4.9	25.4 ± 4.9	25.4 ± 4.9	25.4 ± 4.9	25.4 ± 4.9
3	—	28.1 ± 7.6	24.6 ± 4.7	—	—	—
5	28.0 ± 10.6	26.7**	20.9**	16.0**	23.7 ± 12.9	22.6 ± 2.7
10	26.3 ± 15.0	2.1 ± 0.7	1.7 ± 0.3	2.0 ± 0.4	5.3**	25.0 ± 20.0
15	2.1**	1.4 ± 0.7	1.0 ± 0.4	1.1 ± 0.6	3.4 ± 1.9	26.0 ± 19.1
20	—	1.0 ± 0.3	—	—	—	—
23	—	—	—	Zero***	—	—
25	0.6 ± 0.3	0.7 ± 0.3	Zero***	—	—	—
35	—	0.1 ± 0.1	—	—	—	—
37	—	—	—	—	Zero***	—
40	—	Zero***	—	—	—	—

* Each datum is the mean ± standard deviation (in weeks) of 18–20 pupae, and was derived from a plot of the individual responses on probit paper.

** Bimodal distributions. The mean characteristic of the majority of the sample is recorded.

*** Development is initiated under conditions of constant exposure to the temperatures noted.

plete by the tenth week at 6° C. Yet it will be observed that after the tenth week of chilling a slow downward trend continues in the lower horizontal limb of the curve in Figure 4. Thus, after 35 weeks at the low temperature, adult development begins after only one day at 25° C. And after approximately 40 weeks, adult development actually begins at 6° C. It is equally clear, however, that the changes after the tenth week are extremely slow compared to those occurring during the first ten weeks at 6° C. Moreover, there are substantial reasons, to be considered below, for separating the early and the late effects and attributing the gentle slope of the lower horizontal part of the curve to secondary changes within the fully "accelerated" pupa.

4. *Potentiation of development by temperatures other than 6° C.*

We have considered up to this point the potentiation of development by prior exposure to 6° C. The same technique permits one to examine the latent effects

of any other temperature within the physiological range. The temperatures selected for study in this manner were 2½, 10, 15, 20 and 30° C., the effects in each case being judged in terms of the time subsequently required for the initiation of adult development at 25° C. In order to facilitate comparisons with the results at 6° C., unchilled diapausing pupae of the same 1953-brood were utilized and all experiments were set up synchronously. The over-all results, including those previously described for 6° C., have been summarized in Table III.

In Figure 5 the data have been computed on a rate (1/Time) basis and plotted as a series of asymmetric, bell-shaped curves. Each curve records the rate with

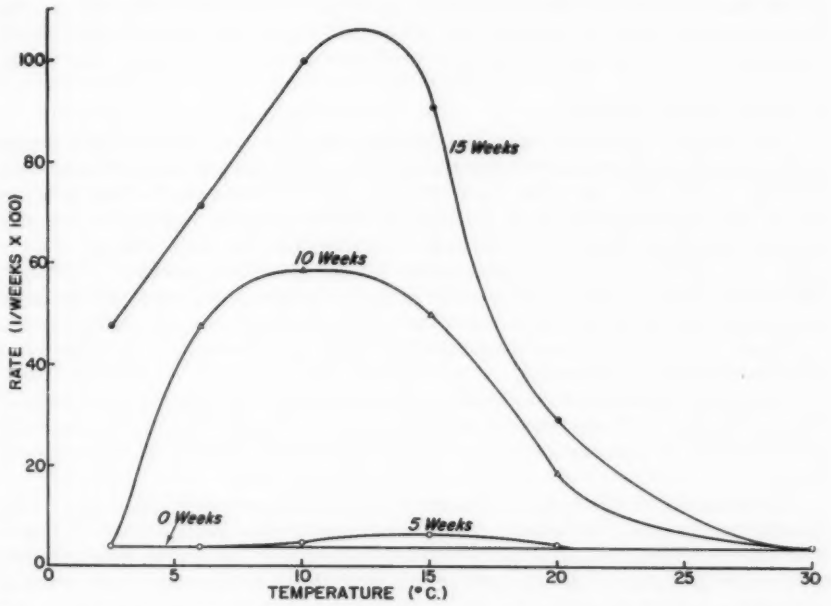

FIGURE 5. The rate (100/Weeks) of initiation of adult development at 25° C. after preliminary exposure to 2½–30° C. for 0–15 weeks.

which adult development is initiated at 25° C. after a particular period of preliminary exposure to temperatures ranging from 2½ to 30° C. For comparison, the corresponding rate in the absence of any preliminary treatment has been recorded as the straight line paralleling the X-axis.

Exposure to any of these temperatures for less than 5 weeks is without detectable effects on the subsequent rate of development at 25° C.—a conclusion already noted in the experiments at 6° C. After 5 weeks of the preliminary treatment, the distribution of results becomes bimodal at 6, 10, and 15° C. (see Table III). In Figure 5 this fact is not evident in the 5-week curve because the plotted points describe the primary mode in each of the three bimodal distri] ations. However, the

bimodality is of interest in itself because it signals the onset of a period of rapid change.

This inference is confirmed in the results recorded after 10 weeks of preliminary treatment. Here a pronounced potentiation of development is apparent after exposure to 6–15° C. And, here also, the results at 20° C. become bimodal (Table III). A continuation of this trend is evident after 15 weeks of exposure, when, for the first time, the range of effective temperatures is broadened to include 2½° C. And, finally, as recorded in Table III, adult development actually begins by the 25th week of continuous exposure to 10–15° C.

The results as a whole are therefore in general agreement with the analysis suggested in connection with the experiments at 6° C. It is of particular interest that an intermediate range of temperatures (6 to 15° C.) is far more effective in potentiating development than are temperatures lower than 6° C. or higher than 15° C.

5. *Effects of brain removal*

The analysis, up to this point, has focussed attention on a physiological change within the diapausing insect which mediates the catalytic effects of low temperature. As long as ten years ago there were already substantial reasons for localizing the site of this physiological change in the pupal brain. It was found that low temperature alters the pupal brain in such a manner that the brain regains its competency to secrete a hormone prerequisite for the initiation of adult development (Williams, 1946). It was also possible to show that the secretion of this hormone is necessary until adult development actually begins. Consequently, if one removes the pupal brain prior to the initiation of development the pupa is stabilized in a state of "permanent diapause."

Manifestly, these previous findings have an intimate bearing on the results of the present study. If the action of low temperature is mediated solely by the brain, then it should be possible to "excise" the effects of prior chilling by removing the brain.

Experiments of this type have been performed on a considerable scale during the past ten years. The results can be summarized most briefly in the statement that among several thousand chilled pupae from which the brains were removed prior to the initiation of development, not a single individual escaped from diapause and initiated development.

Consider, for example, pupae chilled for 35 weeks at 6° C. Though such animals, as illustrated in Table III, initiate development after only one day at 25° C., development is permanently blocked if the brain is removed during those first 24 hours. Such brainless animals continue to live at 25° C. for up to two years thereafter, and during this period cannot be distinguished morphologically from brainless unchilled pupae. By this and analogous procedures it was possible to confirm the fact that the effects of prior chilling can be eliminated by excision of the brain.

Whereas it was initially thought that the brain is no longer necessary after the earliest indication of adult development (Williams, 1946), more detailed studies showed that the brain continues to be required during the first 24 hours of adult development, and, indeed in rare individuals, during the first 48 hours (Williams, 1952). It is of particular interest that excision of the brain during the first 24 hours blocks the further progress of development. Dissection of such animals

showed that the epidermis (which had already loosened its attachments to the pupal cuticle and begun to retract) re-appressed itself to the cuticle and re-established its extremely intimate connections. In effect, therefore, the insect over a period of several weeks returns to a state indistinguishable by any morphological sign from that of a diapausing pupa.

An occasional animal from which the brain had been removed during the second day of development showed an anomalous response in that development of certain specific regions, including head, thorax, wings, legs, and antennae, continued for a time and then stopped with these parts in a more or less advanced stage of adult differentiation. But in virtually all cases where brain removal was postponed until after the first 48 hours of adult development, the latter continued to yield brainless but otherwise normal moths.

6. Quantitative changes in brain activity during chilling

The experiments just considered point to the pupal brain as the vehicle by which low temperature catalyzes the termination of diapause. Under this circumstance one would anticipate systematic changes in the brain's endocrine activity to account for the quantitative aspects of the low temperature effect; *i.e.*, for such changes in development potential as one observes in Figures 4 and 5.

The most detailed study of this sort was performed in 1953–1954 in synchrony with the above-mentioned temperature studies and on aliquot samples of the same homogeneous group of animals. At the outset of the experiment the brains were removed from ten unchilled individuals and tested for activity by implanting them under a plastic window at the tip of the abdomen of a corresponding number of brainless unchilled pupae. Subsequently, at intervals of one to several weeks, the brains of additional 6° C.—animals were removed and tested in like manner. By this procedure it was possible to quantitate the endocrine activity of each implant in terms of the time which it required to evoke the development of the host at 25° C.

The results are summarized in Table IV. It is evident that only an occasional brain was active when obtained from pupae chilled for less than 5 weeks; moreover, in such instances, development ordinarily began only after a considerable delay (up to 40 weeks). By contrast, brains chilled for 5 weeks or longer were not only active in a high proportion of cases, but also, with rare exception, evoked the initiation of adult development within a month after implantation. Evidently, on or about the eighth week at 6° C., the brain attains maximal activity, as judged by the high percentage and uniform rapidity of the responses. Presumably, the occasional inactivity encountered after the eighth week was caused by injury to the brain during the latter's excision and transplantation.

It is particularly fruitful to compare the change in the brain's activity with the previously described behavior of intact pupae after systematic exposure to 6° C. To this end, the mean values in Table IV have been plotted as the broken-line curve in Figure 4. The degree of congruity between these curves leaves little doubt that the abrupt shift in the developmental potential of the pupa as a whole is the outcome of an equally abrupt shift in the endocrine activity of the brain.

Notwithstanding the substantial correlation between the two curves in Figure 4, one also observes minor but highly significant differences. Thus, after chilling for less than 5 weeks, only about one brain in three was found active when tested by

implantation. Yet, even in the absence of chilling, the undisturbed brains of normal pupae exert their effects during the first year at 25° C. This is one of the few differences encountered between the endocrine activity of "loose" implanted brains and brains retaining normal nerve connections. However, such connections are clearly not obligatory since briefly chilled implants were able to achieve threshold activity in one-third of the cases.

A second difference between the curves in Figure 4 is the fact that 50 to 70 per cent of excised brains showed full activity when tested after 5 or 6 weeks of chilling, whereas only about 25 per cent of unoperated pupae developed promptly after the same amount of chilling. Since this period coincides with the time of

TABLE IV

Tests of endocrine activity of brains from pupae previously chilled at 6° C. for specific periods*

Prior chilling of brains (weeks)	Number tested	Number active	Time** to evoke development at 25° C. (weeks)
0	10	2	10; 11; – —
3	10	4	3.3; 3.3; 23.9; 40.6; —
4	10	3	2.7; 3.0; 37.9; —
5	20	14	3.7 ± 1.1
6	10	5	4.6 ± 1.9
8	10	7	2.7 ± 1.3
10	10	10	2.3 ± 0.6
12	10	7	2.9 ± 1.1
15	10	10	2.0 ± 0.9
19	16	16	2.0 ± 0.7
25	8	8	2.1 ± 0.6
35	10	9	1.4 ± 0.3
44	9	8	2.4 ± 1.7
45	22	19	2.9 ± 1.1

* Each brain was implanted into a brainless diapausing pupa and the time for the latter to initiate adult development at 25° C. determined.

** Mean ± standard deviation in weeks are recorded for all groups in which at least 50 per cent of animals developed.

rapid change in brain activity, conceivably the difference is a sampling artifact attributable to the limited number of animals tested. However, it is even more likely that the difference is real and that, by some unknown means, the surgical manipulation of the brain during this critical period hastens to completion the changes which restore the brain's endocrine activity.

A final difference between the two curves in Figure 4 concerns the lower segments after the tenth week of chilling. It is clear that after 8–10 weeks at 6° C. the brain has fully regained its endocrine powers and that only minor changes occur thereafter. By contrast, in normal unoperated pupae the onset of development at 25° C. is progressively accelerated when the low temperature treatment is prolonged beyond the tenth week; indeed, we have already observed that adult development actually begins at 6° C. after about 40 weeks. This implies that a slow release of the brain hormone takes place at the low temperature and that a threshold titer

is ultimately built up after about 40 weeks at 6° C. Consequently, after 35 weeks at 6° C. the titer is barely sub-threshold and further brain function at 25° C. is required for only one day. Since the excised brains were tested in brainless unchilled pupae lacking any substantial titer of brain hormone, one would anticipate the observed difference between the lower segments of the curves in Figure 4.

On the basis of these various observations, the effects of low temperature on the intact diapausing insect are subject to interpretation. Low temperature promotes a transformation of the brain so that the latter becomes competent to secrete its hormone; as illustrated in Figure 4, this process is complete by the tenth week at 6° C. The slow secretion of the hormone then begins at the low temperature. Once the brain has regained its endocrine powers, then, as illustrated in Figure 1, the secretion of the hormone is actually retarded by low temperature. The catalytic effects of low temperature on the diapausing pupa may therefore be said to end as soon as low temperature has induced the physiological transformation of the brain.

7. In vivo and in vitro chilling of the brain

In the course of the routine preparation of several thousand brainless diapausing pupae used in the studies of the present series, the excised unchilled brains were often implanted into brainless diapausing hosts and then subjected to chilling under conditions of *in vivo* culture. In this manner it was possible to build up a "brain bank" which was useful for various purposes. Such brains, after a suitable period of chilling, could then be reclaimed and utilized as active chilled brains.

This finding, in itself, gives assurance that the normal connections of the brain with the rest of the nervous system are not prerequisite for the temperature effect. However, as Andrewartha (1952) has pointed out, the experimental method did not exclude the possibility that low temperature acts indirectly on the brain—perhaps by making available within the host some precursor of the brain hormone.

Under this latter circumstance one would expect the hypothetical activating substance to be present in high concentration in pupae chilled for a prolonged period. This prospect was tested by excising the brains of previously chilled pupae and reimplanting one or more brains of unchilled pupae. In no case did the previously chilled host have any detectable effect in activating the unchilled implant. Manifestly, this result argues against the view that low temperature acts by promoting the release of some brain-stimulating factor within the pupa as a whole. It also opposes an alternative possibility that low temperature destroys a brain-inhibitor within the pupa as a whole.

A conclusive test of the direct action of low temperature on the brain obviously requires that the brain be activated apart from the rest of the insect; *i.e.*, under conditions of *in vitro* culture. This objective is not easy to realize in view of the unsatisfactory state of knowledge concerning the culture of insect tissues other than blood cells and spermatocytes (Schmidt and Williams, 1953).

Nevertheless, during the past ten years a considerable number of unchilled brains have been subjected to *in vitro* culture under sterile conditions, the media consisting of various types of physiological solutions or of blood obtained from chilled, unchilled, or developing animals. The blood-containing media invariably underwent darkening during prolonged exposure to low temperature, notwithstanding the presence of crystals of the potent anti-tyrosinase, phenylthiourea. Darkened

blood is known to be extremely toxic to insect tissues, and such experiments were doomed to failure.

In no case did the brains survive the low temperature culture for longer than about two weeks. Such brains, when implanted into brainless pupae, were immediately attacked by phagocytic cells and ultimately broken down.

One might suppose that even dead brains, if sufficiently numerous, might contribute a critical concentration of hormone after implantation. This possibility was discounted by the finding that active brains, killed by freezing and thawing in liquid nitrogen or by exposure to temperatures higher than 50° C., were ineffective when implanted in large numbers. Evidently, only a trace of hormone is stored in the normal active brain, since the biological effect of a single hemisphere of a living brain cannot be duplicated by the implantation of fifty dead brains.

Consequently, for reasons that are believed to be technical in character, it has been impossible to accomplish this final and most direct test of the view that low temperature achieves its catalytic effect by its direct action on the brain. However, the sum total of available evidence gives little reason to think otherwise.

8. *Winter sickness*

It will be recalled that the onset of adult development under conditions of constant exposure to 6° C. is delayed until approximately the fortieth week at the low temperature. Even then, the development which takes place fails to proceed beyond an early stage, corresponding to that attained after only two days of adult development at 25° C. If the exposure to 6° C. is continued still longer, one detects the onset of a curious syndrome for which we suggest the name "winter sickness." The distinctive feature is a progressive paralysis of the intersegmental muscles of the abdomen; *i.e.*, the muscles responsible for the "tone" of the motile abdominal segments and the latters' characteristic circular motion in response to mechanical stimulation.

In pupae returned to room temperature after 46 weeks at 6° C., the tone of these muscles is subnormal for several days thereafter, and contraction can be evoked only when the pupa is subjected to mechanical stimulation such as squeezing. Further prolongation of the low temperature treatment leads to a flaccid paralysis of all muscles save the heart. As summarized in Table V, electrical excitability likewise disappears, and contraction can no longer be induced in response to tetanizing shocks administered to the abdomen by overlying electrodes.

The early stages of winter sickness are reversible at room temperature until about the fifteenth month at 6° C. (see Table V). After several days at 25° C., electrical excitability returns, followed shortly therafter by motion in response to mechanical stimulation, and, finally, by the recovery of tone. Adult development then continues where it had left off. However, if the exposure to 6° C. has been for longer than a year, the adult moths, when formed, are generally feeble and unable to spread their wings, or, indeed, to escape from the pupal exuviae. And in the case of female individuals only a few eggs are matured.

After 15 months at 6° C., winter sickness becomes irreversible for a progressively larger number of individuals. Such animals show the absence of heart beat when returned to room temperature, and, in fact, are already dead, as judged by the lack of any detectable oxygen consumption.

Winter sickness is not peculiar to the Cecropia silkworm, since it has been regularly encountered in all species of diapausing pupae which we have studied at low temperatures. For example, *Telea polyphemus* shows reversible sickness after 60 weeks at 6° C. and death after 90 weeks; *Samia walkeri* (*cynthia*) shows flaccidity after 35 weeks at 6° C. and death after 90 weeks. *Antheraea mylitta* and *Actias selene* are even more susceptible, being killed by 6 months at 6° C.

A considerable number of experiments were performed in search of the physiological basis of winter sickness. Of particular interest was the paralysis of the abdominal muscles—a condition which one fails to duplicate even by the injection of such potent agents as curare or botulinus toxin.

TABLE V

Winter sickness after prolonged exposure to 6° C.
(Platysamia cecropia)

Weeks at 6° C.	No. pupae	Stage of adult development after chilling	Behavior at 25° C.
42	40	0–1 day	Lively; prompt development
44	20	0–1	Lively; prompt development
46	25	0–1	Induced motion only; prompt recovery and development
49	40	0–1	30% show flaccid paralysis and electrical inexcitability at outset; all recovered and developed
52	50	0–1	50% flaccid and inexcitable at outset; all recovered and developed
57	90	0–1	70% flaccid and inexcitable at outset; all recovered and developed into feeble moths
66	40	0–2	All flaccid and inexcitable at outset; only 60% recovered and developed into feeble moths
70	65	0–2	All flaccid and inexcitable at outset; only 50% recovered and developed into feeble moths
74	10	0–2	All flaccid and inexcitable at outset; only 25% recovered and developed into feeble moths
90	50	0–2	All flaccid and inexcitable; none recovered

It was first thought that winter sickness might be peculiar to animals showing early adult development. This prospect was negated by the finding that at 2½° C., in the absence of any trace of adult development, the onset of winter sickness is even more prompt than at 6° C. This same effect was noted in other species. Indeed, in *Samia walkeri* (*cynthia*) winter sickness becomes irreversible after only 7 months at 2½° C.

A clue as to the physiological basis of winter sickness was provided by the following experiment performed on *Samia walkeri* (*cynthia*). From seven individuals showing reversible winter sickness the blood was drained and exchanged with that of a corresponding number of normal diapausing pupae. All animals were then maintained at 25° C. Within 12 hours the normal pupae receiving the winter-sick blood showed full and complete paralysis of the abdominal muscles. Electrical excitability also disappeared and reappeared only 3–5 days later. Recovery was synchronized with the formation of a chalky precipitate, which settled

on the plastic window at the tip of the abdomen and also deposited itself in the lumen of wings, antennae, and the body cavity as a whole.

The winter-sick pupae which received normal blood likewise recovered in 3–5 days, though whether this was hastened by the transfusion of normal blood cannot be stated in the absence of suitable controls. No precipitate formed in this blood and none was detected in the adult moths that ultimately formed.

In effect, therefore, it was possible passively to transfer the winter sickness by the transfusion of winter-sick blood. Moreover, the detoxification of this blood was accompanied by the formation of a copious white precipitate, presumably containing ureates and other insoluble end-products of nitrogenous metabolism.

Samia walkeri (*cynthia*) is a species which never escapes from diapause if maintained at temperatures higher than 20° C. After 15 months at 25° C., the normal, lively pupa shows the formation and deposit of the same sort of precipitate as mentioned above. Evidently, the intermediate metabolism of the pupa at the warm temperature is able to cope with nitrogenous end-products by detoxification and "excretion by precipitation," whereas, at temperatures below 10° C., the production of soluble end-products outbalances their detoxification. Winter sickness, in this sense, appears to be a genuine case of auto-intoxication within the "closed system" of the pupal insect, and to bear a resemblance to the mammalian syndrome of uremia. The phenomenon obviously merits further study.

Discussion

On the basis of the experimental results set forth in the preceding sections, it is clear that the catalytic effects of low temperature on the diapausing insect are mediated by an endocrine mechanism. This mechanism centers in the brain. Previous studies had already demonstrated that the brain loses its endocrine powers just prior to the pupal moult and is inactive in the freshly pupated insect (Williams, 1946, 1952). We now see that the rate with which the brain recovers its activity is largely determined by environmental temperature.

By an apparently direct action on the brain itself, temperatures within the range 6 to 15° C. greatly accelerate the functional recovery of the brain. As soon as the brain has undergone this physiological change, the catalytic effects of low temperature are at an end. The pupa, equipped with an endocrinologically competent brain, becomes, in effect, the equivalent of a non-diapausing pupa.

It is fruitful to consider the similarities between two such animals; *i.e.*, between chilled diapausing pupae and unchilled non-diapausing pupae. Thus, in both types of animals the actual secretion of the brain hormone, as well as the development that follows, are favored by high temperature and retarded by low temperatures. Moreover, in both types of animals one can ordinarily establish or re-establish a pupal diapause by prompt excision of the pupal brain (Williams, 1952). And, finally, in both types of pupae, low temperature is still capable of enforcing a developmental standstill which is reversible at higher temperatures. On the basis of this comparison it seems necessary to conclude that the end-point of the pupal diapause is physiological in character, and is signalled by the functional recovery of the brain.

If attention is now directed to the overwintering of the diapausing insect, it is clear that exposure to temperatures within the optimal range of 6 to 15° C. ordinarily continues beyond the 10 weeks required for the full and complete recovery

of the brain. Hence, under normal circumstances, the dormant state begins as a "diapause" and continues thereafter as a "quiescence" (Andrewartha, 1952; Lees, 1955). The net effect is that the overwintering insect is poised to react to the warm temperatures of spring.

From the physiological point of view, attention focusses on the brain as the vehicle of the low temperature effect. Indeed, within the substance of the pupal brain, it is reasonable to pin-point the temperature effect even more closely within the 26 neurosecretory cells which secrete the brain hormone. Low temperature apparently accelerates the functional recovery of these cells from their inactive state in the diapausing pupa.

The activating reaction, itself, pursues a typical time-course as dictated by temperature. Critical exposure to low temperature is first required, following which the activation proceeds to completion within a period of several weeks. The unknown reaction within the neurosecretory cells therefore shows the kinetics of a threshold reaction but not of an "all or none" reaction.

We have observed that an intermediate range of low temperatures (6 to 15°C.) is optimal for the physiological transformation of the brain. At temperatures as high as 25° C. or as low as 2½° C. the process is greatly retarded. The fact that 2½° C. is far less effective than 6° C. gives assurance that no single temperature-inhibited reaction (such, for example, as adsorption) will suffice. The minimal requirements appear to be the interaction of two antagonistic reactions with different temperature coefficients, the one favoring and the other opposing the activation of the neurosecretory cells.

Until very recently, the analysis could not be pursued beyond this point. Most fortunately, however, the biochemical and physiological events within the pupal brain of the Cecropia silkworm have just been described in detail by Van der Kloot (1955). In brief, the unchilled diapausing brain is found to be not only endocrinologically incompetent, but also electrically silent—a state which can be accounted for in terms of the apparent absence of cholinesterase from the brain as a whole. The cholinergic substrate of this enzyme (presumed to be acetyl choline) is, at the outset, present in only trace amounts. Exposure to low temperatures greatly accelerates the accumulation of this substrate within the brain. Then, when the chilled pupa is returned to 25° C., the brain shows a prompt, and, presumably, inductive synthesis of cholinesterase. The titer of acetyl choline decreases to an intermediate level, and electrical and endocrine activities immediately reappear. These various changes are peculiar to the brain and are not encountered in any of the other ganglia.

The implications of these extraordinary findings are discussed in detail by Van der Kloot. For present purposes it is of particular interest that the entire brain is shut down at the outset of diapause and reactivated months later under the influence of environmental temperature. What this means in terms of the function of the neurosecretory cells is not clear. Van der Kloot suggests that the entire brain is inactivated to prevent the neurosecretory cells from being driven by nerve impulses at synapses with afferent and internuncial neurons. And one may likewise conjecture that within the neurosecretory cells the synthesis and secretion of hormone continues to require cholinergic mechanisms of the same type as preside over the genesis of nerve impulses and the release of neurohumors.

The primary change induced by chilling therefore appears to be the synthesis and

accumulation within the brain of a cholinergic substrate which appears to be acetyl choline. It will be recalled that molecules such as acetyl choline are notoriously unstable in solution at physiological pH, and one may predict that the accumulation of substrate is opposed by non-enzymatic breakdown. Consequently, it is even possible that the synthesis and breakdown of the cholinergic substrate correspond to the pair of antagonistic, temperature-sensitive reactions whose existence was found necessary to account for the endocrinological activation of the brain.

Summary

1. The termination of the pupal diapause of the Cecropia silkworm is potentiated by preliminary exposure to low temperature and, especially, to temperatures in the range 6 to 15° C.

2. This effect is mediated by an endocrine mechanism which centers in the brain, and is specifically concerned with restoring the competency of the brain's neurosecretory cells to synthesize and secrete the brain hormone.

3. The time required for the initiation of adult development at 25° C. is thereby reduced from 27 weeks to as little as one day.

4. As soon as the brain has recovered its endocrine function, the catalytic effects of low temperature are at an end; the actual secretion of the hormone and the developmental response that follows are then favored by high temperature. In this manner the low temperatures of winter potentiate the initiation of adult development the following spring.

5. The effects of low temperature on the inactive, diapausing brain are considered in detail. The action is apparently a direct one on temperature-sensitive reactions within the brain itself. The over-all process of activation shows the kinetics of a threshold reaction but not of an all-or-none reaction.

6. These findings are discussed in the light of Van der Kloot's chemical and physiological studies of the brain of Cecropia. Within the substance of the brain the primary change induced by low temperature appears to be the synthesis and accumulation of a cholinergic substrate.

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